SECOND ANNUAL AQUATIC TOXICITY WORKSHOP

1975 PROCEEDINGS

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G.R. Craig

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INTRODUCTION

The first Aquatic Toxicity Workshop was held in August 1974 at the Environment Canada Freshwater Institute in Winnipeg where toxicity laboratories across Canada were represented. The main purpose was to describe the different facilities and compare the technical approaches to bioassay testing of industrial wastes and microcontaminants. The concensus of opinion was that meetings of this type were valuable in the development of bioassay methodology and should be repeated on an annual basis. It was generally agreed that each year a different laboratory would host the Workshop and its continued success would be dependant on the participation of laboratories across Canada.

This year a number of specific topics were chosen for discussion and presentations were made by Canadian industries, consultants, universities and regulatory agencies and one university from the United States.

Records of informal workshops of this type frequently remain unpublished. We feel that benefits to other workers in the field are thereby lost and consequently the proceedings have been compiled for this Workshop and hopefully the resources and time will be available to continue this practice.

I would like to acknowledge the time and effort that went into the preparation and co-ordination of the Workshop by the Ministry of the Environment Toxicity Unit staff. Particular appreciation is extended to Mrs. M. Barclay and Mrs. J. Kurdel who typed and retyped the discussion sections of this text and contributed considerable time in the hidden clerical tasks that made this Workshop a success.

The 1976 Aquatic Toxicity Workshop will be sponsored by Environment Canada E.P.S. at the Halifax laboratory, notification of which appears at the end of the proceedings. I am sure that it will be every bit as successful and well attended as this year's Workshop and will encourage other agencies to host future conferences.

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TOPIC I

STANDARD BIOASSAY METHODOLOGY

FISH STOCKS - BIOASSAY USE

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November, 1975

Abstract

There has been a definite need in bioassay testing to standardize both the holding facilities and the fish stocks involved. Both our previous and present water systems were evaluated as to the quality of treated water produced. An evaluation was also carried out on the laboratory fish holding facilities. In order to achieve greater control over the fish used in testing programs the feasibility of hatching our own stocks was examined. Availability of fish eggs from reliable sources was determined for a year round testing program. A study of our hatching, rearing and holding facilities and procedures was conducted. The fry were analyzed as to the time from hatching until they reached experimental weight. A comparison of the quality of laboratory hatched fish versus hatchery fish was carried out using the reference chemicals Sodium Lauryl Sulphate and Sodium Pentachlorophenate.

Introduction

Since the opening of the Aquatic Toxicology Laboratory in Edmonton, there has been a definite problem with maintaining a proper fish loading density in our experiments. Loading density can be defined as the volume of bioassay sample per gram of fish over a period of time. The cause of this problem has been the size of the fish we have been receiving from the hatchery. We were also concerned about the physical condition of the fish (tolerance, high mortality and abnormal or deformed fish). It was decided to hatch and raise our own fish in order to maintain better control over our fish stocks. In this way we would have fish of a more suitable mean weight and therefore, better control over loading densities. This hatching of our own fish was also necessary as our present source of rainbow trout fry would be unavailable after December, 1975. In order to check the quality of the laboratory hatched fish, they were tested against our existing stock of hatchery fish using reference chemicals (EPS Report 1-WP-74-1 and Davis and Hoos).

Methodology

In order for us to hatch our own eggs, the water treatment system was analyzed and upgraded for improved water quality. After bringing in the first shipment of eggs and hatching them, we realized that our water treatment system would have to be improved if we were planning to continue hatching eggs for our supply of test fish. At the time of the first shipment of eggs, the water treatment system included: CO_2 injection for pH control, 2 five gallon per minute anthrafilt filters for suspended solids removal, 2 ultra violet units for water disinfection and 4 five gallon per minute activated carbon filters for chlorine removal. With this system the maximum flow was approximately 8 gallons per minute. This flow was not sufficient so we upgraded the system to its present status: CO_2 injection, 1 thirty-five gallon per minute anthrafilt filter, 1 thirty-five gallon per minute activated carbon filter, a 1000 gallon water reservoir equipped with a 7½ horsepower refrigeration/heating unit for temperature control, a sodium thiosulphate drip system for final chlorine removal and 1 ultra violet unit. This system will give a flow of approximately 35 gallons per minute. A summary of the chemistry of the city and laboratory treated water is as follows:

Parameter	City Water	Treated Water
pH	9.0 - 9.5	7.4 - 7.8
Temperature (°C)	8.0 - 16.0	10.0 ± 2.0
Total Free Chlorine (mg/ℓ)	--	< 0.01
Total Residual Chlorine (mg/ℓ)	3.00 - 4.00	< 0.01
Hardness (mg/ℓ CaCO$_3$)	70 - 100	70 - 100

Twenty thousand certified disease free rainbow trout eggs were brought in from an American hatchery and placed into 4 incubator trays. The eggs were in the eyed stage of their development and hatched within 7 to 10 days after their arrival. The water flow through the incubators was approximately 8 litres per minute. An analysis of the water was done twice a day. The eggs were sorted twice a day and any dead or deformed eggs were removed from the trays. There was approximately 5% mortality during the incubation period.

Upon hatching, the fish were placed in fry troughs. An attempt was made to set up a fast current in these containers by maintaining a water flow of about 18 litres per minute. The fry were held in the troughs until they weighed between 0.5 and 1.0 grams. There was about 20% mortality in the fry troughs over six weeks.

When the fry reached the weight of 0.5 to 1.0 grams, they were moved to 200 gallon acclimation/holding tanks. When new high flow, low volume tanks arrived, the fish were transferred to them. The mortality in these tanks was approximately 10% over five months.

The hatchery fish were placed in the 200 gallon acclimation/holding tanks directly after their arrival. When the new tanks arrived, they were placed into them. The average mortality in these fish during the holding period has been about 40% to 50% for the last few shipments. Before these shipments the mortality was between 10% to 20% for the holding time.

While the fish were held in the hatching, rearing and holding facilities, the water chemistry was monitored twice daily for dissolved oxygen, pH, total and free residual chlorine, temperature and flow rates. The dissolved oxygen was always maintained at greater than 7.0 mg/ℓ. The pH was kept between 7.40 and 7.80 and there was less than 0.01 mg/ℓ free and total residual chlorine. At one time the total residual chlorine rose to about 0.04 to 0.06 mg/ℓ but the addition of the sodium thiosulphate drip system reduced this to a non-detectable level. The water temperature was maintained at about 10°C except in the temperature acclimation tanks where immersion heaters kept the temperature at 15°C. The fish were held at this temperature for two weeks prior to testing. The flow rates in the various types of tanks were as follows: the incubators 8 to 10 ℓ/min, the fry troughs 10 to 20 ℓ/min, the 200 gallon acclimation/holding tanks 5 to 6 ℓ/min and the high flow, low volume holding tanks 10 to 14 ℓ/min.

While the fish were below 1.5 grams in weight, they were fed a diet of commercial trout food at a rate of 4% to 6% of their body weight. When they reached 1.5 grams they were fed a maintenance diet of about 2% of their body weight per day. These amounts were fed to them over two feedings per day. The maintenance diet was to ensure that the fish were healthy but was low enough to suppress any rapid gain in weight. This procedure ensured fish of a suitable weight over extended periods of time. The fish were not fed for two days before being used in any experiment.

Results and Discussion

It was deemed necessary to test the new fish against the fish we had been receiving for the past two years to ensure experimental continuity. Therefore, they were tested for their tolerance to two standard chemicals, Sodium Lauryl Sulphate and Sodium Pentachlorophenate. The tolerance testing began on August 1, 1975 for the Sodium Lauryl Sulphate and on August 20, 1975 for the Sodium Pentachlorophenate. Five different tests were conducted with each chemical using both the laboratory hatched fish and the hatchery fish. All of the tests were conducted in constant temperature water baths using 40 litres as the test volume. All of the tests were static bioassays with no test solution replacement. Five chemical concentrations plus controls were used for each test except in the August 1 tests on Sodium Lauryl Sulphate where four concentrations plus controls were used.

The bioassays conducted on the Sodium Lauryl Sulphate were 24 hours in duration. Mortality was checked and recorded at ½, ½, 1, 2, 4, 8, 12 and 24 hours. The chemical parameters pH, dissolved oxygen and conductivity were monitored at 0 and 24 hours and the temperature was checked at 0, 12 and 24 hours. There was no aeration in any of the concentrations. The concentrations used for the five tests were as follows:

TEST NUMBER	TEST DATE	CONCENTRATIONS (ppm)
1	1/8/75	6.0, 4.0, 2.0, 1.0, control
2	13/8/75	5.0, 4.0, 3.0, 2.0, 1.0, control
3	20/8/75	5.0, 4.0, 3.0, 2.0, 1.0, control
4	17/9/75	5.0, 4.5, 4.0, 3.5, 3.0, control
5	19/9/75	5.0, 4.5, 4.0, 3.5, 3.0, control

Twenty-four hour LT$_{50}$ values were determined wherever possible (Litchfield). The 24 hour LC$_{50}$ values were derived either by plotting LT$_{50}$'s versus concentration (Sprague) or by plotting concentration versus per cent survival (Litchfield and Wilcoxin). An LC$_{50}$ range was also reported for each test. The LC$_{50}$ results can be summarized as follows:

Hatchery Fish

TEST NUMBER	LC$_{50}$ RANGE (ppm)	LC$_{50}$ VALUE (ppm)
1	2.0 - 4.0	3.00
2	4.0 - 5.0	4.35
3	2.0 - 4.0	3.52
4	4.5 - 5.0	4.55
5	4.0 - 4.5	4.39

The mean LC_{50} value for the hatchery fish was 3.96 ± 0.45 ppm.

Laboratory Hatched Fish

TEST NUMBER	LC_{50} RANGE (ppm)	LC_{50} VALUE (ppm)
1	2.0 - 4.0	3.00
2	4.0 - 5.0	4.15
3	4.0 - 5.0	4.20
4	4.0 - 4.5	4.20
5	4.0 - 4.5	4.20

The mean LC_{50} value for the laboratory hatched fish was 3.95 ± 0.28 ppm.

The results of the 24 hour static bioassay on Sodium Lauryl Sulphate comparing the two stocks of rainbow trout show a negligible difference between the LC_{50} values for the hatchery fish versus the laboratory hatched fish. The mean LC_{50} for the hatchery fish was 3.96 ± 0.45 ppm while the mean LC_{50} for the laboratory hatched fish was 3.95 ± 0.28 ppm. The laboratory hatched fish appeared to show less variation in their response to the chemical. The values for both of the fish stocks easily fall within the range of 1.0 to 10.0 ppm as suggested in the EPS Report 1-WP-74-1 (1974).

The Sodium Pentachlorophenate was prepared according to the procedure of Alderdice (1963). The experiments were 96 hours in duration. The concentrations for all the bioassays were 1.00, 0.50, 0.10, 0.05 and 0.01 ppm plus controls. Mortality was absserved and recorded at ¼, ½, 1, 2, 4, 8, 12, 24, 36, 48, 60, 72, 84 and 96 hours. Chemistry checks were performed on pH, conductivity and dissolved oxygen at 0, 24, 48, 72 and 96 hours while temperature was monitored at 0, 12, 24, 36, 48, 60, 72, 84 and 96 hours. The concentrations were aerated to maintain a dissolved oxygen concentration of greater than 7.0 mg/ℓ. The results can be summarized as follows:

Hatchery Fish

TEST NUMBER	TEST DATE	LC_{50} RANGE (ppm)	LC_{50} VALUE (ppm)
1	20/8/75	0.05 - 0.10	0.09
2	28/8/75	0.10 - 0.50	0.15
3	9/9/75	0.10 - 0.50	0.12
4	17/9/75	0.10 - 0.50	0.12
5	17/9/75	0.10 - 0.50	0.12

The mean LC_{50} value was 0.12 ± 0.00 ppm.

Laboratory Hatched Fish

TEST NUMBER	TEST DATE	LC_{50} RANGE (ppm)	LC_{50} VALUE (ppm)
1	20/8/75	0.05 - 0.01	0.08
2	28/8/75	0.10 - 0.50	0.11
3	9/9/75	0.10 - 0.50	0.10
4	17/9/75	0.10 - 0.50	0.12
5	17/9/75	0.10 - 0.50	0.11

The mean LC_{50} value was 0.10 ± 0.00 ppm.

The results of the 96 hour bioassays using Sodium Pentachlorophenate show very little difference between the two stocks of fish. The mean LC_{50} for the laboratory hatched fish was 0.10 ± 0.00 ppm while the fish from the hatchery were slightly more tolerant and had a LC_{50} of 0.12 ± 0.00 ppm. These values compare favorably with the range of 0.037 to 0.130 ppm as reported by Davis and Hoos (1975).

Conclusions and Recommendations

It would seem that raising fish from eggs is practical for various reasons. After the initial outlay for the cost of incubators and other necessary holding tanks (approximately $600.00 to $1000.00) and after the first hatch is at a useable weight, then it is less expensive to purchase and ship eggs as opposed to fish. For 20,000 rainbow trout eggs the cost is about $100.00. There was approximately 60% to 65% survival in our first hatch to useable weight. There was unusually high mortality due to the chlorine problem mentioned before and also due to the problems encountered with the fry troughs. We were unable to set up a fast current in the troughs which could have possibly been responsible for the high mortality while the fry were held in these. Theoretically, 85% to 90% survival should be achieved when hatching eggs. The cost for 20,000 rainbow trout fry would be about $1000.00. With the high mortality we have been experiencing in the past (about 50%) this works out to about $100.00 per thousand fish. Therefore, excluding shipping costs, it is more economical to bring in eggs rather than fish.

With the hatching of our own eggs it is easier to control the size of the fish. They can be acclimated for bioassay use from the time that they reach 0.5 grams (about 3 to 4 months) until they reach 2 to 3 grams (about 8 to 10 months). These weights allow for a suitable loading density for static bioassays with the equipment presently in use at the laboratory. Therefore, for from 3 to 10 months following hatching, suitable fish from one stock are available for use in bioassays.

Rainbow trout eggs from a certified disease free hatchery are usually available twice a year. By bringing in two shipments of 30,000 eggs, spread about six months apart, there should be about 50,000 fish (based on 85% survival) for use in biological testing. This makes available about 950 fish per week which is more than adequate for the present needs of the laboratory.

The results of the tolerance testing indicated that the fish raised in the laboratory were comparable to the hatchery fish used for the previous two years. Therefore, it is feasable for a laboratory to hatch and raise their own fish in order to have an adequate and useful stock of standardized rainbow trout for their bioassay testing.

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IMPORTANT CONSIDERATIONS IN ACUTE AND SUBLETHAL BIOASSAY PROCEDURES FOR WATER QUALITY STUDIES WITH AQUATIC LIFE: A REVIEW

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INTRODUCTION

Toxicity bioassays constitute one of the most commonly used biological methods in aquatic environmental studies in the world today. There is a vast literature dealing with the subject and excellent reviews have appeared which attempt to compile and summarize progress in the field (Herbert, 1965; Edwards & Brown, 1967; Alderdice, 1967; Burdick, 1967; Warner, 1967; Beak, 1958; Wuhrmann, 1952; Marchetti, 1962; Sprague, 1969, 1970, 1971, 1973).

Sprague (1969) in particular, makes a plea for standardization of terminology and use of detailed and accurate test procedures with a view to making bioassay results more reliable, comparable and universally interpretable. Hopefully, one of the results of toxicity workshops like the present one will be to provide a better understanding of current methology, short comings and factors influencing aquatic bioassays in use in Canadian laboratories. Ideally, we should soon be able to standardize a set of procedures for Canadian useage which offer accuracy, sensitivity, reliability and simplicity for water quality useage.

The objective of this paper is to summarize, in the author's opinion, important considerations in the choice, design and usefulness of both acute and sublethal bioassay procedures. Admittedly, some of the examples given here have only been tested with kraft pulp mill waste which has been our major research interest for several years. However, the concepts discussed should be applicable to a wide range of pollutants.

Types of Bioassays. A wide choice of bioassay procedures are available to the investigator depending on the experimental goal of that investigator. Essentially, the bioassay is a test which assesses the response of a living organism to some factor - for our purposes, the response to the presence of a toxicant or stress-producing circumstance such as high temperature or low oxygen. The choice of test type, test organism and sophistocation of technique should be based on the answers desired by the investigator. Do we wish to assess the potential toxicity of an industrial discharge to receiving waters inhabited by important recreational or commercial species? Is a comparison of relative toxicity of various waste streams desired? Do we merely wish to monitor variations in toxicity of a specific discharge with time? Thus, the first step in conducting a bioassay must be to accurately define one's experimental goals and select the test procedure that best applies. One frequently gets the impression that certain procedures are dogmatically applied without much consideration of the reliability or usefulness of the result. Obviously, a standard LC50 test conducted with 1 species under laboratory conditions is difficult to relate to a complex food chain in the environment where biological interactions are poorly understood and physical and chemical characteristics of the environment vary considerably with time. Unfortunately, the above crude tests are often the only tools available and one must make use of them in lieu of better methods. The important point to remember is not to put too much faith in the results of such tests - ie. to remember their limitations both in design and application. One often gets the impression that people interpret a bioassay result as "gospel" and "etched in stone forever".

Basically there are two types of bioassays in current useage-acute and sublethal bioassays. Acute tests yield information on the concentration and time course producing a lethal response in a test organisms. Sprague (1969) discussed the various terminology in use to define the results of such tests and recommended the use of the term LC50. The LC50 is the concentration of toxicant lethal to one half the test organisms at some specified time - usually

96 hours. Sprague is careful to point out that acute mortality frequently does not cease within 96 hours and that one must ascertain whether the toxicity curve has become assymptotic to the time axis prior to terminating the test. The point at which the toxicity curve does become assymptotic to the time axis approximates the incipient LC50 (the concentration where 50% of the test organisms just start to die regardless of how long the exposure to toxicant). Figure 1 shows curves for boron toxicity (from sodium metaborate) to Pacific salmon in both fresh and saltwater. Obviously, if tests had been terminated at 96 hours, or some other arbitrary time, a far different and less sensitive result would have been obtained. Furthermore, figure 1 shows that toxicity in fresh and salt water differ markedly indicating that one should not assume uniformity of results between different media.

A variety of acute toxicity test procedures are available (Table 1). Simple static tests can be used where the test organisms are exposed for some time in tanks of test substance without solution renewal. Well known objections to this method involve the potential for less of volatile toxicants from solution, adsorbtion of toxicants onto the walls of the container or the body surface of test organisms, extraction of toxicant by test organisms resulting in a gradual decrease in solution toxicity with time, or "treatment" of the toxicant via the metabolism of the test organism itself. All these factors would tend to decrease the apparent toxicity of the solution tested (David & Mason, 1973). Conversely, fish waste products may build up in solution increasing toxicity or permitting the transmission of disease. Obviously, such tests have many disadvantages which render them poor for accurate or prolonged useage. Conversely, they are simple and economical of test solution and of manpower, once setup. In my opinion these tests are perfectly adequate for screening procedures or preliminary studies provided that the results are obtained

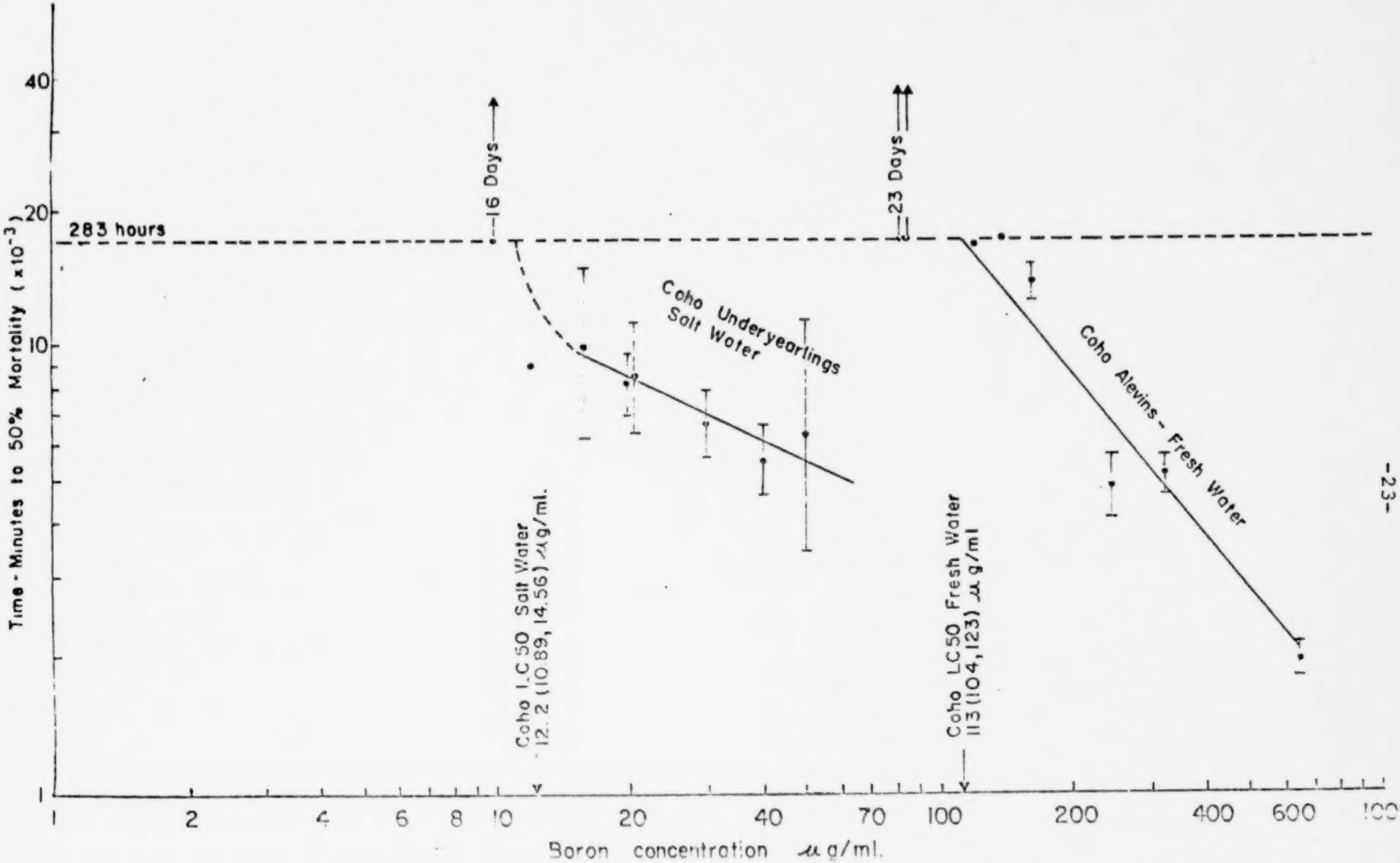

Figure 1 - Toxicity of boron to coho salmon underyearlings and alevins in fresh and salt water. Note the long time course of the bioassay and the large difference in incipient LC50 for the fresh and saltwater groups. The 95% confidence limits are given for the LC50's.

Table 1 . TYPES OF BIOASSAY TESTS

ADVANTAGES	DISADVANTAGES	OPTIMUM USE
	STATIC TEST	
-SIMPLE, INEXPENSIVE -ECONOMICAL OF TOXICANT AND MANPOWER -WELL DOCUMENTED	-DETOXIFICATION POSSIBLE -WASTES ACCUMULATE -VOLATILES EASILY LOST	-TEST < 12 HOURS -COMPARATIVE TESTS*
	STATIC WITH REPLACEMENT	
-SIMPLE, FAIRLY ACCURATE -FAIRLY ECONOMICAL OF TOXICANT	-REQUIRES REGULAR ATTENTION -ORGANISMS STRESSED DURING REPLACEMENT?	-ROUTINE LC50'S -TESTS ≤ 96 HOURS
	CONTINUOUS FLOW	
-MOST ACCURATE -NO DETOXIFICATION OR WASTE BUILD-UP -GOOD METHODS AVAILABLE	-COMPLEX APPARATUS -HIGH TOXICANT USE -SPACE REQUIRED	-RIGOROUS LC50's -TESTS > 96 HOURS -FOR MAXIMUM ACCURACY
	IN SITU	
-INCLUDES NATURAL CONDITIONS -CAN USE LOCAL SPECIES -NO EFFLUENT REQUIRED	-DIFFICULT TO OBSERVE AND MONITOR -ORGANISMS STRESSED DURING OBSERVATIONS? -DIFFICULT TO CONTROL AND INTERPRET	-RECEIVING WATER SCREENING TEST -TESTING INTERACTIONS UNDER NATURAL CONDITIONS

quickly - ie. within about 12 hours. Such tests can be usefully employed for toxicity comparison purposes where median survival time data are compared using solutions of moderate to high toxicity where death is rapid.

A refinement of the static acute toxicity bioassay constitutes the static test with solution replacement. In this test, part or all of the toxicant solution is replaced regularly with fresh solution, overcoming at least in part, many of the disadvantages of the static test without replacement.

Perhaps the most accurate form of acute toxicity test, is the continuous flow procedure. In this procedure toxicant is continually replaced by an inflow of new solution by some automatic delivery system (Marchetti, 1962; Stark, 1967; Abram, 1960; Grenier, 1960; Mount & Brungs, 1967; Mount & Gaddum, 1953; Engstrom-Heg, 1971; Granmo & Kollberg, 1972; Maciorowski & Kondra, 1975). Sprague's (1969) guidelines for a flow equal to volume in 3 - 5 hours and a solution volume of 2 - 3 l/g of fish/day are highly recommended for continuous flow procedures. Continuous flow tests overcome most of the deficiencies of static tests and usually result in an estimate of greater apparent toxicity (Loch & MacLeod, 1974). For example, G.M. Kruzynski (in Rogers, *et al*, 1975), obtained a 96 hr LC50 of 0.5 mg/l dehydroabietic acid, a resin acid found in pulp mill waste, with sockeye salmon yearlings (*Oncorhynchus nerka*) by continuous flow procedures compared to 1.3 - 2.2 mg/l 96 hr LC50 by static test procedures. Furthermore, Kruzynski (in Davis & Hoos, 1975a, b) showed that approximately 89% of the theoretical dehydroabietic acid was actually present in solution as determined by gas chromatography of water samples in the theoretical concentration range of 2.0 - 0.63 mg/l in continuous flow bioassays. This finding emphasizes the need for workers to measure the actual concentration of toxicant in the test tank during the bioassay to avoid making errors in apparent concentration.

Continuous flow tests, when properly designed and carried out, offer the most accurate form of acute toxicity test and should be used for rigorous experimental or regulatory work. They do have serious disadvantages however, in that the dosage apparatus is often complex and expensive and requires considerable attention. In addition, large volumes of test solution are required which is a great practical problem when testing effluent of relatively low toxicity such as pulp mill waste. Obviously, for routine monitoring purposes or for toxicity comparisons, a simple practical test is required.

Figure 2 illustrates the relationship between coho underyearling survival time in full strength bleached kraft mill effluent and the 96 hr LC50 established by rigorous 96 hour bioassay procedures. Such a graph, which has been prepared for effluent from 2 coastal B.C. pulp mills, allows one to predict the 96 hr LC50 from survival time data with a simple test that is completed within 3 - 24 hours. Such a test might prove useful for a variety of toxicants as a simple monitoring procedure. Similarly, the residual oxygen bioassay (Carter, 1962; Ballard & Oliff, 1969) where organisms are sealed in jars and their ability to depress the dissolved oxygen is assessed in the presence of a toxicant, may provide a rapid, sensitive test procedure that is economical of effluent. Currently, two laboratories on the West Coast have submitted interesting papers for publication based on this procedure.

Acute toxicity bioassays have been used in the field where they may be termed in situ acute toxicity tests. Usually a basket or net full of test organisms is lowered into receiving waters to a specific depth and observed periodically for mortality. A number of problems are inherent in this procedure which include choice of test organisms (native or laboratory stock), cycling physical and chemical conditions in the water which are difficult to

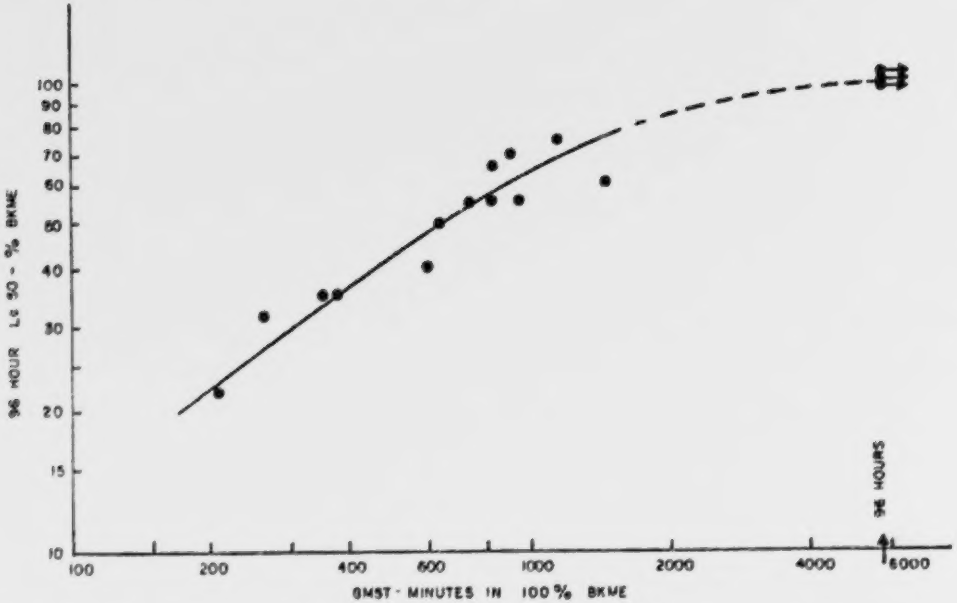

FIG. 2 The relation of the geometric mean survival time calculated from mortality in full strength BKME and the 4-day LC50 determined for a number of effluent samples. Data are for underyearling coho salmon, 0.8–14 g, in fresh water 20 ppm total hardness as $CaCO^3$, 11.1±0.2 C at a loading density of 0.56±.06 liter/g.

monitor precisely, inability of test organisms to behave naturally due to the confines of the chamber and lack of a real control on the experiment. In my opinion, *in situ* acute tests are useful to give an indication of a problem in receiving waters but are rather difficult to interpret accurately and control precisely.

Comparisons of Toxicity Tests - Reference Toxicants

When one tries to compare bioassay results from the literature it is evident that the variety of procedures used, test organisms employed, and variations in methodology make comparisons very difficult and potentially a questionable practice. Sprague (1969) pointed out the need to report results and procedures in detail, including the water chemical and physical characteristics and statistical information accompanying the derivation of the LC50. In an excellent review, Lee (1973), showed the chemical considerations important in comparing bioassay results.

One technique that enables more reliable comparisons of results involves the use of reference toxicants. The principle is to expose the test organisms to standard toxicants and record results from this exposure along with results of tests with other toxicants. In this way inherent differences in test conditions and sensitivity of different test stocks will be evident and hopefully, results of different laboratories be made more comparable. Davis & Hoos (1974;1975) tested 2 reference toxicants, dehydroabietic acid and sodium pentachlorophenate in a co-operative study involving seven British Columbia laboratories. Results (Table 2) showed that there was fair consistency of findings between the laboratories and that differences, in some instances, could be explained by variations in physical and chemical characteristics of the bioassay. They concluded that reference toxicants would detect large differ-

TABLE 2 Results of static bioassays and summary of test conditions reported by the various laboratories coded in a random fashion. LC50s were calculated in two ways, as a log-probit estimate or as an estimate resulting from a nomographic analysis yielding 95% confidence limits for the LC50 (upper and lower limits in brackets). The slope function, S, for the nomographic analysis is given. Fish condition factor, C.F., was calculated using the mean fish weight and length given in the table for each test.

	LC50									
Lab. code	Log-probit estimate	Nomographic calc. + 95% CL	S	Loading density (g/l)	$\bar{x}$ initial temp-C	$\bar{x}$ initial pH	$\bar{x}$ hard-ness ppm	$\bar{x}$ wt g	$\bar{x}$ length cm	C.F. Wt/L^3
		rainbow trout — ppb sodium pentachlorophenate								
E	92	98(87.5, 109.8)	1.136	0.52	12.0	6.96	51.5	2.84	5.98	1.33
D	96	96(90.1, 102.2)	1.109	0.42	11.54	7.0	47.0	0.87	4.28	1.11
C	50	50(29.8, 84.0)	1.99	0.48	11.0±0.1	7.0±0.1	5-6	1.90	5.80	0.97
B	100	106(88.5, 127.0)	1.331	≃0.50	11.5	7.02	5.0	1.52	5.24	1.06
A	48	47(32.9, 67.2)	1.732	≃0.50	10.04	5.7	4.0	1.39	4.84	1.23
F	75	–*	–	0.58	10.0±1.0	7.0±0.1	10.0	1.31	–	–
		rainbow trout — ppm dehydroabietic acid								
E	1.03	1.06(0.99, 1.14)	1.116	0.54	10.94	6.98	23.0	3.33	6.32	1.31
D	1.05	–	–	0.57	12.25	6.97	47.0	1.26	–	–
C	1.22	–	–	0.48	11.0±0.1	7.0±0.1	5-6	1.90	5.80	0.97
B	1.74	1.85(1.62, 2.11)	1.237	≃0.50	11.5	6.92	5.0	1.40	5.32	0.93
A	1.18	1.20(1.03, 1.39)	1.118	≃0.50	9.3	9.0	4.0	1.67	4.96	1.37
F	1.25	–	–	0.57	10±1.0	7.0±0.1	10.0	1.29	–	–
		coho salmon — ppb sodium peniachlorophenate								
E	96	92(79.3, 106.7)	1.12	0.76	10.25	7.01	14.7	4.61	7.40	1.14
C	37	31.8(24.9, 40.6)	1.317	0.52	11.0±0.1	7.01±0.1	5-6	2.14	5.80	1.10
		coho salmon — ppm dehydroabietic acid								
E	1.38	–	–	0.69	10.62	6.97	7.0	4.18	6.24	1.72
C	1.76	1.75(1.56, 1.96)	1.134	0.52	11.0±0.1	7.1±0.1	5-6	2.14	5.80	1.10
		sockeye salmon — ppm dehydroabietic acid								
D	1.38	–	–	0.46	11.56	7.07	47.0	1.70	–	–
G	2.14	2.23(2.03, 2.45)	1.114	0.45	7.5	7.7	85.0	1.38	4.92	1.16
		sockeye salmon — ppb sodium pentachlorophenate								
D	50	50(40.3, 62.0)	1.281	0.56	12.5	7.19	47.0	0.68	–	–
G	130	130(119, 142)	1.096	0.46	7.5	7.70	85.0	1.38	5.05	1.07

*Data insufficient for calculation of confidence limits.

ences in sensitivity of stock fish (ie. - those weakened by chronic disease) and that guidelines for toxicity tests should contain the use of reference toxicants as a means of standardizing test results.

Alderdice (1963) selected sodium pentachlorophenate as a suitable "toxic stressor" based on a list of properties that should apply to all reference toxicants:

- "High solubility in water
- virtually complete ionization in solution at pH 7.0 or higher (excepting organics)
- stability in solution at pH 7.0 - 8.5
- availability as a dry solid of high purity
- facility for quantitative determination
- toxicity to fish (in low concentrations)"

Ideally, a range of reference toxicants should be available which are selected from a list of major pollutants such as pesticides, detergents, heavy metals, wood-processing effluent and petrochemical wastes. Also useful, might be upper lethal temperature tests (Howard, 1973). Virtually any stressor should serve as a useful tool to detect abnormally weak or insensitive stocks, however care should be exercised in the choice of reference test as one may be inadvertently testing for acclimation phenomena when using temperature or sodium chloride as reference stressors.

Sublethal Effect Studies

Sublethal effect tests comprise another form of bioassay in which the response is not death but some change in the physiological, behavioral or developmental processes of an organism in the presence of a toxicant. As such, they are more sensitive than acute toxicity tests as they show the concentration of stressor that produces a response in the animal in a more subtle, yet potentially equally damaging way. These responses occur at concentrations often well below those which are acutely toxic and thus there is an incipient sublethal response threshold which defines the "no effect" level for the stressor. It is my feeling that definition of the incipient sublethal response threshold for a variety of sublethal effects will give us the best indication of values useful for receiving water criteria definition. For example, a recent literature survey (Davis, 1975) indicated that sublethal effects of kraft pulp mill waste are evident in salmonid respiration, circulation, growth, behavior, metabolism, swimming energetics, temperature tolerance and hematology. Table 3 summarizes these effects and their incipient threshold concentrations. Such summaries can form the basis of possible dilution criteria for minimizing sublethal effects on fish populations (Table 4). The same sort of procedure could be applied to a wide range of pollutants.

A useful concept contained in Tables 3 and 4 is the expression of sublethal responses as some fraction of the acute toxicity value. Expressing results in this way is useful as it allows consideration of a biological response index rather than a simple physical dilution factor. Also, this method allows effluents of varying toxicity to be compared more easily as it is a form of biological standardization. Finally, the method makes the simple acute toxicity test a useful predictive tool for assessing concentrations likely to cause sublethal effects, once the interrelation between acute and sublethal thresholds is known. Thus, acute tests could serve as monitoring tools to minimize sublethal effects when the "application factor" for the incipient sublethal threshold is known. Obviously, many of the procedures and apparatus employed for sublethal tests are too complex and time consuming for practical monitoring purposes. Criteria, such as those in Table 4, must be considered tentative and subject to revision with new knowledge and should

TABLE 3 - SUBLETHAL EFFECTS OF NEUTRALIZED COMPOSITE WHOLE KRAFT MILL EFFLUENT ON SALMONIDS

Species	Function or System Affected	Nature of Response	Fish Size	Test Temp.	Conc. Tested	Sublethal Threshold Concentration	Comments	Reference
Salmo gairdneri	Respiration	Elevated "cough" frequency	20.3-25.4 cm	11 ± 1	-	0.08 96 hr LC50	Response reduced after 2 hrs.	Walden, *et al.*, 1970
" "	Circulation	Reduced arterial O_2 sat'n.	150 g	10	0.33 96 hr LC50	-	No adaptation after 24 hrs.	Davis, 1973
Oncorhynchus nerka	Respiration	Elevated "cough" frequency	207 - 321 g	10.5 ± 0.5	-	0.1-0.2 96hr LC50	Response reduced overnight	" "
" "	"	Ventilation volume up	" " "	" "	-	0.2 96hr LC50	" " "	" "
" "	"	Oxygen uptake increased	" " "	" "	-	0.33 96hr LC50	" " "	" "
" "	Growth	Growth rate and food conversion efficiency decreased	2.4 - 2.8 g	15	–	0.08 96hr LC50	After 8 weeks exposure	Webb & Brett, 1972
" "	Behaviour	Fish avoid effluent	3 cm	10	-	0.2 96hr LC50	Within 1 hr exposure	G.M. Kruzynski[1]
Oncorhynchus kisutch	Circulatory	White blood cell count thrombocyte count reduced	juvenile	11 ± 1	-	0.1 TL_m 96	After 21 days exposure	McLeay, 1973
" "	"	Blood neutrophil count elevated	"	11	-	0.25 TL_m 96	After 200 days exposure	McLeay & Brown, 1974
" "	Metabolism	Liver glycogen depressed	9.0 ± 0.4 g	11	0.8 LC50	-	1/5 of initial level after 72 hours exposure	McLeay & Brown, 1975
" "	"	Plasma Glucose elevated	" " "	11	" "	-	3 fold increase after 96 hrs exposure	" " "
" "	"	Elevated plasma glucose & lactate, decreased liver & muscle glycogen	" " "	11	0.7 LC50	-	Following exercise & post-exercise recovery in effluent	" " "
" "	"	Plasma glucose elevated	juveniles	11	-	0.10 TL_m 96	After 200 days exposure	Howard, *et al*, 1972

[1] personal communication

TABLE 3 - (continued)

Species	Function or System Affected	Nature of Response	Fish Size	Test Temp.	Conc. Tested	Sublethal Threshold Concentration	Comments	Reference
Oncorhynchus kisutch	Metabolism.	Blood and Muscle lactate elevated	juveniles	11±1		0.25 TL_m96	After 200 days exposure	Howard *et al*, 1972
" "	"	Muscle glycogen depressed	"	"		0.10 TL_m96	" " " "	" " "
" "	Energetics	Swimming ability impaired	"	13±0.5		0.20 TL_m96	Exercise in test chamber	Howard, 1973
" "	Temperature Tolerance	Reduced upper lethal temperature	"	10		0.062-0.23 TL_m96	Lethal temperature bioassay	" "
" "	Respiratory	Elevated cough frequency	post smolts	10		0.148 TL_m96	Evidence of adaptation	" "
" "	Growth	Growth rate enhanced	4-10 g	10-13		0.1-0.2 96hr LC50	After several weeks exposure	Mason & Davis, 1975
" "	"	" " "	juveniles	12±1		0.25 96hr LC50	200 day exposure	McLeay & Brown, 1974
" "	Behaviour	Feeding less vigorous	4-10 g	10-13		0.1-0.2 96hr LC50	Response lasted 2 weeks & then disappeared	Mason & Davis, 1975
" "	"	Fish slow and "unresponsive"	"	12-13		0.15 96hr LC50	After several weeks exposure	" " "
Salmo salar	Behaviour	Fish avoid effluent	7.7-14.8	17±0.2		Approx. .006 96hr LC50	"Vague" response	Sprague & Drury, 1969

Table 4 - TENTATIVE DILUTION CRITERIA FOR NEUTRALIZED WHOLE BKME TO MINIMIZE SUBLETHAL EFFECTS ON SALMONIDS

CONCENTRATION	EFFECT
0.1 96 hr LC50	- SUBSTANTIAL INCIDENCE OF SUBLETHAL EFFECTS
0.05 96 hr LC50	- SOME SENSITIVE EFFECTS MAY BE PRESENT (LOWERED UPPER LETHAL TEMPERATURE, COUGHING, AVOIDANCE BEHAVIOUR)
0.02 96 hr LC50	- ABSENCE OF MOST SUBLETHAL EFFECTS (0.006 96 hr LC50 PRODUCES "VAGUE" AVOIDANCE IN ATLANTIC SALMON)
BELOW 0.02 96 hr LC50	- TAINTING OF FLESH ? - COLOUR EFFECTS ?

never be considered inviolate.

Factors Influencing Acute and Sublethal Bioassay Results

Numerous factors affect the outcome of bioassay tests and many have been summarized by Sprague, (1969, 1970, 1971, 1973) and Lee (1973) as well as other workers. Particularly important are:

a) Chemical Aspects

Results may be affected by oxidation state, solubility, complexation, salt ratios and concentrations, organic content, ionic strength, and presence of solids, in tests. These factors must be considered when applying results of controlled laboratory studies to receiving water, as well as the physical characteristics of the water. Measurements of the chemical species studied should be made by analysis of bioassay solutions during the test to assure uniformity and accuracy of doseage. Chemical characteristics accompanying the test such as water hardness, pH, salinity, and dissolved oxygen should be reported for each test.

b) Sample Collection, Handling, Oxygenation and Storage

Large differences in results can result from collection, handling and storage methods applied to samples. Obviously the desired sample should be representative of "typical" conditions although this is often difficult to obtain or ascertain with highly variable effluents such as pulp mill waste. In some samples, toxic volatiles are easily lost (Werner, 1963; Hynninen, 1971) or aeration in the bioassay itself may reduce toxicity (David & Mason, 1973). To combat these problems some workers conduct tests in a pure oxygen atmosphere (Ozburn, *et al*, 1973) or adopt gentle aeration with pure oxygen (Loch & MacLeod, 1974). Another approach has been to preaerate the sample to drive off volatiles and conduct the test with the residual persistent toxicants (Davis & Mason, 1973). The rationale behind this latter procedure is that persistent toxicants are likely to cause most of the problems in well mixed receiving waters and volatiles will rapidly be lost under such conditions.

Some effluents, such as pulp mill waste, undergo detoxification during storage (Howard & Walden, 1965; Servizi *et al*, 1966; Webb & Brett, 1972; Werner, 1963) and detoxicification may be rapid at elevated temperatures (Sprague & McLeese, 1968a, b). In our experience, the effect of storage on kraft mill effluent is unpredictable (Davis & Mason, 1973) and toxicity may decline, change little,or occasionally increase. For the above reasons, whenever possible, storage and transport time of samples should be minimized and tests conducted with fresh samples. If some delay is necessary, samples should be stored at low temperature. Ideally, on-site testing in portable facilities (Lake & Loch, 1973) offers the best chance of minimizing storage and handling effects.

c) Fish Loading Density

A very important factor affecting bioassays is the loading density, expressed in weight of fish/unit volume of test solution. Sprague (1969) recommended use of 2 - 3 L/g of test fish (changed daily) as the minimum loading density permissable in bioassays. Use of higher loading densities is economical of toxicant but often reduces the apparent toxicity of the solution. Figure 3 shows how survival time of sockeye salmon underyearlings is enhanced at loading densities below 2 L/g test fish. Clearly, Sprague's recommendations should be carefully followed in all types of toxicity tests. One possible exception is a very rapid test such as the Carter (1962) residual oxygen bioassay where death may occur from hypoxia and poor O_2 extraction before loading density affects the results.

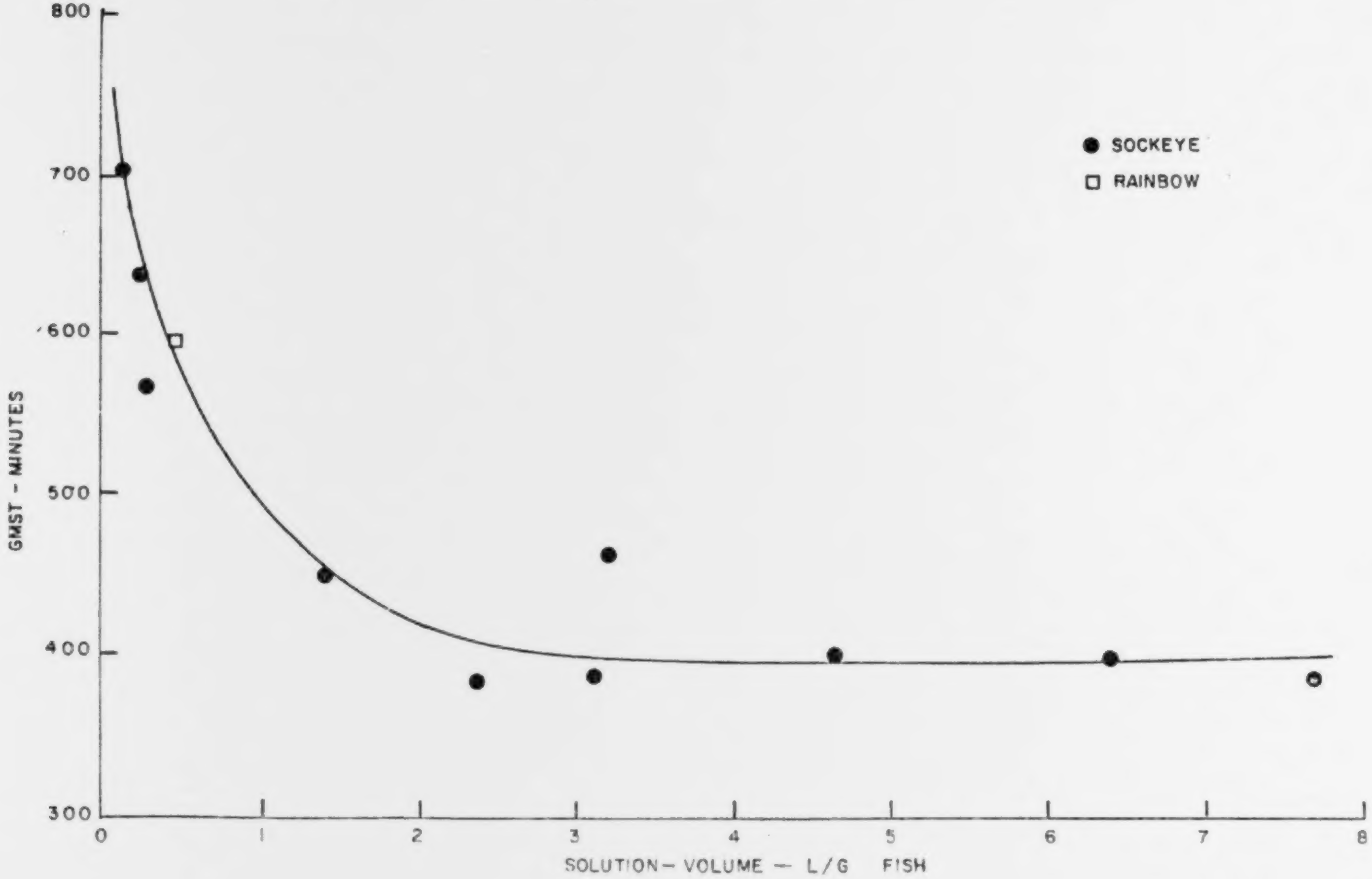

Figure 3 - The effect of fish loading density on survival times of sockeye and rainbow underyearlings in a highly toxic bleached kraft pulp mill effluent. GMST is geometric mean survival time (see Davis & Mason, 1973).

d) Animal Size, Species and Condition Factor

It is not particularly clear how animal size affects results of bioassay tests in much published work. Generally embryos and larvae appear particularly sensitive to various stresses and research with these forms is proving particularly productive. Woelke (1962, 1967, 1972) has demonstrated particular sensitivity with developing Pacific oyster larvae to toxicants and new procedures such as byssal thread production in *Mytilus* are being evaluated (Salazar, 1973). One suspects that many freshwater and marine invertebrates important in major aquatic food chains will prove to be sensitive test organisms. Most fish work involves studies with young specimens, such as underyearling salmonids, and few studies have compared results with different age and size groups. Servizi, *et al*, (1966) observed that young sockeye salmon appeared more resistant to bleached kraft pulp mill effluent than mature sockeye although conditions of exposure for the two groups varied somewhat making the comparison questionable. Davis & Mason (1973) found no correlation between size, weight and sensitivity to kraft mill waste in coho salmon ranging from 0.24 - 4.05 g and 2.0 - 7.3 cm, although too small a range was likely examined. That study showed that only fish with a condition factor,CF, calculated:

$$CF = 100\ (wt/leng.^3)$$

where: wt = weight in g

leng. = fork length in cm

below 0.8 appeared more sensitive to toxic stress. This makes sense as thin "weakling" fish would be expected to succumb readily. Thus, it seems wise not to use fish with condition factors below 0.8 in toxicity tests.

A range of sensitivity can be expected between different species when exposed to a specific toxicant. In our laboratory, we have not definitely established that any one salmonid species is more sensitive to toxicants than any other and suspect that apparent differences may relate to the general health and state of development of test stocks. Some preliminary work indicated that the 3 spine stickleback, *Gasterosteus aculeatus*, appeared more resistent to pulp mill waste than coho salmon underyearlings although far more work must be done on comparative sensitivity of species. Table 5 shows some examples of varying species sensitivity to 2 samples of neutralized, filtered full bleach kraft mill waste.

It seems best that toxicity studies make use of sensitive, representative organisms that are known to inhabit the receiving water in question. Ideally, tests should be done with species collected in the test area or at least with genetic strains common to the area. If such procedures are not practical, some means must be found of varifying the reliability of tests done with other stocks of organisms. Sensitive procedures with invertebrates, especially those that can be followed through several generations, may prove useful in future.

In Summary, let us consider a list of specific points identified as important in both acute and sublethal test procedures. Hopefully, future work and guidelines will consider many of these points:

1. The first step in choosing a bioassay procedure is to accurately define the goals and objectives of the test. Once this is done, the test procedure should be selected which gives the most reliable and accurate information. Limitations of the procedure must be considered in the application of results.
2. Two classes of test type are available - acute and sublethal bioassay procedures using a variety of organisms. Acute toxicity tests include the simple static variety, static with solution replacement, and continuous flow procedures. Each has its merits and limitations and the choice

Table 5 -

TOXICITY OF KRAFT PULP MILL EFFLUENT TO VARIOUS MARINE ORGANISMS

(2 batches of neutralized, filtered effluent, salinity corrected, if required).

Species	Response	Test Duration-hours	Concentration (% full strength)
BATCH 73-H			
Coho underyearlings	Death - LC50 result	96	36%
Coho underyearlings	Sublethal threshold	rapid	3.6%
Pelagic copepod	Death - LC50 result	118	10%
Amphipod - *Anisogammus pugettensis*	break-down of copulatory behavior	approx. 24	15-20%
Polychaete *Capitella capitata*	No apparent effect	96	100%
BATCH 74-A			
Pink alevins	Death - LC50 result	96	37%
pink alevins	sublethal threshold	rapid	3.7%
Amphipod *Amisogammarus pugettensis* (adults)	break-down of copulatory behavior	approx. 24	30-40%
A. pugettensis (juveniles)	Death - LC50 result	96	10%

depends upon the answer required and degree of accuracy desired. *In situ* tests are useful for receivng waters but difficult to monitor and control precisely.

3. Acute toxicity tests provide answers in terms of toxicant lethality to test subjects. Sublethal tests are more sensitive and can yield information on incipient sublethal threshold concentrations. Such tests are often complex and lengthy and sometimes of little practical value for routine useage. If the relationship between acute and sublethal response is known, the acute response may serve as a predictive tool for estimating sublethal response thresholds. For this reason, it is useful to express sublethal responses as some fraction of the LC50.
4. Simple, rapid toxicity tests are required that are sensitive and economical of test sample. Residual oxygen bioassays or assays similar to the oyster larval bioassay may prove useful.
5. Much more work is required with organisms other than fish. Organisms selected from lower trophic levels that constitute important members of aquatic food chains may be particularly valuable.
6. Toxicity tests can be more easily compared by using reference toxicants. Such toxicants will help identify varying sensitivity in test stocks. Desirable properties of reference toxicants include high water solubility, complete ionization at pH 7.0 or higher (excepting organics), stability in solution at pH 7.0 - 8.5, availably as a high purity, dry solid, facility for quantification, and high toxicity.
7. Chemical and physical characteristics affecting bioassays must be considered and reported with results. Of importance are oxidation state, solubility, complexation, salt ratios and concentrations, organic and solid content, and ionic strength. In addition, water hardness, pH, oxygen

content, salinity and temperature must be considered and reported. Toxicant presence and concentration should be confirmed by quantitative analysis of bioassay solutions during the test.

8. Results can be markedly affected by effluent collection, storage and handling procedures. Samples should be representative and be stored for a minimum of time, preferably at low temperature. On site testing with fresh samples will provide the most accurate results for unstable samples. Aeration of effluent may result in loss of volatiles and detoxification.
9. Fish loading density in all tests should not exceed 2 - 3L/g fish (changed daily) in all but very rapid tests. Higher loading densities will usually result in underestimates of toxicity.
10. A range of sensitivity to toxicants is to be expected between test species. Choice of test species should be carefully made. No clear relationship of fish size or age and sensitivity to toxicants is evident except in the case of embryos and larvae which are often sensitive. Standardization of size and age of test species will improve comparability of tests. Fish with condition factors below 0.8 should not be used. Work with animals with a short life cycle that can be followed through several generations may prove useful.

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A COMPARISON OF SOME TRADE WASTE SAMPLING TECHNIQUES

by

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In general terms, the most important question to be asked about a liquid industrial effluent is, "What do we want to know about it and with what degree of certainty". Specifically, this question becomes:

1) Is the waste always lethal, frequently lethal, occasionally lethal?
2) Is the waste extremely toxic, moderately toxic, slightly toxic?
3) Can the lethality be reasonably attributed to particular effluent components?

Over the past years the Ministry of the Environment has had the opportunity to study a variety of trade wastes and to ask these questions. These studies have not only answered particular questions but also raised additional ones.

One such study concerned three major sewers from a large petrochemical plant. Initially, static bioassays on grab samples from these sewers produced no mortality to fathead minnows at 100% in 96 hours. None of these sewers had any sort of retention facilities that would modulate flow or slug discharges. Subsequently, exposure tanks were set up downstream of each of these three outfalls and rainbow trout were exposed to river borne concentrations of the wastes for a two month period. The results of this protracted exposure indicated that after initial dilution one of the sewers produced no acute lethality. One of the sewers was subject to frequent and severe fluctuations in pH which ultimately led to the death of the fish and the third sewer was subject to frequent and severe slugs of toxic material such that complete survival never extended more than 23 days. During these slug discharges up to 250 ppm styrene and 80 ppm ethylbenzene were measured in the fish tank. Contaminated water condensed from crude light oil (benzol) also contributed to the slug discharges.

The greatest drawback to this type of project is the lack of control over the effluent concentrations in the exposure tanks. On the other hand, one of the best things about the project is that it provided an instant evaluation of diluted effluent quality such that the plant management was made more aware of the effects of the waste and especially the impact of slug discharges on receiving water quality.

The next study involved an extended bioassay and growth evaluation of rainbow trout exposed to the effluent from an oil refinery. The refinery used in this study was capable of processing 35,000 barrels of crude oil per day. The oily process waste water (400 IGPM) was passed through two A.P.I. separators (in parallel) and then into equalizing basins. The effluent proceeded through a flocculation tank and primary clarifier, then through an activated sludge system. Following secondary clarification, the effluent was subjected to ozonation and discharged to a holding basin where any storm water and surface runoff was added. The mixture of treated waste and storm water was then discharged. This treated waste was not lethal to fathead minnows at 100% over 96 hours of static exposure but was lethal to rainbow trout in a continuous flow bioassay in 96 hours.

The continuous flow bioassays utilized waste which was continuously cooled and proportionally diluted with temperature controlled, unchlorinated water. The mortality of the fish did not appear to be a response to a uniform, steady concentration. Rather, the fish would survive for a period and then those in the highest and a few in the adjacent concentration would die. Mortality would temporarily cease and then recommence at the highest remaining concentration. Ultimately, death was observed as low as 10% and 50% impairment of growth at 5% was observed. The mortality response while undoubtedly related to the concentrations of the toxicant(s) shifted down the effluent dilution scale as each progressively larger slug discharge was emitted.

Positive identification of the lethal component(s) was not possible but the project did demonstrate not only the technical feasibility of such a biomonitoring project but also the variability present in even a highly treated, lagooned waste.

Lastly, a series of grab samples were collected from a sewer in a steel mill. This sewer which drained the coke oven and by-product recovery area was similar to those flows in the first example, in that there was no lagooning of any kind to modify effluent characteristics. The grab samples were pumped directly from the sewer into either a 45-gallon drum or a 500-gallon trailer. These undiluted samples were then tested with fathead minnows and rainbow trout.

The data below illustrates that given the same industrial waste, different test species and/or test methods will produce different answers.

TABLE 1

Bioassay Comparison - Coke Oven Waste

Bioassay Type	Test Species	Result
Static	Fathead Minnow	in 96 hrs.
Static	Rainbow Trout	96 hr. LC_{50} = 60%
Constant Flow	Rainbow Trout	96 hr. LC_{50} = 34%

In considering the results of these examples, a variety of questions arise as to the purpose of the sample and the kind of answer which the test is to provide. The purpose ought to be to provide some information ultimately leading to the definition and the realization of a "safe" industrial waste.

One of the less obvious results of these various bioassay techniques is cost. Table 2 outlines the cost of three types of bioassay assessment. On-site biomonitoring which is an extremely stringent test of effluent quality leaves no room for error on the part of the company. For a regulatory agency on the other hand, it is a very expensive surveillance technique.

TABLE 2

Estimated Bioassay Costs

Type of Bioassay	Man Days	Cost per Day	Total Cost $
Grab Sample* Static Bioassay	4	180	720
Composite Sample C./F. Bioassay	6	180	1,080
Biomonitoring Set-Up	40	200	8,000
Exposure	80	200	16,000

*Assume a crew of two people

These costs are based on a combined, estimated labor cost of $140 per day, food and lodging of $50 per day and travelling of 50 miles per day at 20¢ per mile. The cost includes collection of the sample and production of a final report but does not include any chemical analyses beyond those necessary to run the bioassay ie. temperature, dissolved oxygen, pH and conductivity. If in one sampling run it is possible to collect four to six separate effluent samples, then the cost per bioassay decreases to approximately $200 per bioassay.

Is any sample a good sample? The first and second examples indicated that static bioassays on grab samples produced no lethality. When a more sensitive species was exposed to these wastes under continuous flow conditions these wastes proved to be lethal.

Grab sampling may allow extra ordinary precautions to be taken within a plant. During long term exposure experiments these extraordinary measures cannot be maintained. Should we, as effluent regulators notify the industry as to the time and date of our sampling program or merely tell them that samples will be collected within a particular time period and do they anticipate any major process changes or down time during that period. Does notification really matter if the effluent, as in example 2, is taken from a lagoon of several days holding capacity or even from a discharge pipe flowing at the rate of 50 or 60 million gallons per day. Can the manufacturing processes be sufficiently fine tuned on a short term basis to affect the quality of the effluent? I would suggest that many industrial managers, if given the choice between down time and lost production or trouble with the regulatory agencies over faulty or leaky processing equipment, would choose the latter rather than the former.

Does a composite sample provide a better indication of what the effluent is like? Do we want to know what the average effluent is or do we want to know the worst 10%. We need to know how bad the effluent can get, for how long and how often. A great deal of good, conscientious effluent control work can be undone by a few minutes inattention or a momentary mishap. Is a toxicity profile based upon individual, sequential samples either grab or composite the same as a profile developed through biomonitoring.

Presumably the fluctuations in the quality of an effluent that has been through a waste treatment system will be well moderated as compared to those whose only treatment is travelling down a pipe from process to receiving water. That such moderation should occur is acknowledged in the Federal Guidelines for both the oil refining industry and the pulp and paper industry. The relationship between biomonitoring results and those results obtained through other sampling and analytical techniques and especially the Federal Effluent Guidelines and Regulations have yet to be clarified.

If the purpose of the bioassay sample is a routine 24-hour static toxicity test or a governing 96-hour continuous flow test to establish compliance with the federal effluent regulations and guidelines then the method of sampling, the point of sample collection and the relevant data collection have all been prescribed. Results on effluents sampled consistently in this manner will be comparable within a plant from year to year and with other, similar plants sampled in a comparable manner.

If the purpose of the sample collection is not to establish compliance with the federal documents but to perhaps identify in-plant sources of lethality, then the type, size and location of sample point will have to be specified according to the experimental design.

When consideration is given to factors such as the integration of new samples into the flow of work in the laboratory, acclimation of fish, simplicity of field operations and minimum expense, probably the best compromise sample is one which is collected over a reasonable period with parallel information on flow, production and any recognized process upset. With a backlog of such information accumulated from a number of similar. discrete samples the data can be composited to yield an estimate of central tendency and variability.

The responsibility for the successful implementation of such a program of effluent analysis and improvement rests with the discharger of the effluent. Such an approach will hopefully bring about the realization of a safe effluent while at the same time optimizes the economies of resources, manpower, time and money.

ACUTE TOXICITY OF PULP AND PAPER MILL EFFLUENTS USING DAPHNIA AND RAINBOW TROUT AS TEST ANIMALS.

by

R.J.P. Brouzes and V.A. Naish

ABSTRACT

Several acute toxicity bioassays, using both Daphnia (water flea) and rainbow trout, were conducted on the various effluent streams of a pulp and paper mill. The results indicate a marked difference between the response of Daphnia and rainbow trout to these streams. The possible reasons for these differences are discussed along with the advantage of using Daphnia in acute toxicity bioassays.

Introduction

Domtar has become very concerned about its toxic effluents discharged to the environment. In 1969, under sponsorship of CPAR, Domtar undertook its first toxicity and tainting study. As a result of that study a number of streams were identified as prime contributors to toxicity and tainting. The most toxic streams contained foul condensates which proved to be most efficiently treated by steam stripping (steam distillation) as opposed to biological or physical/chemical treatment. This study from 1969 - 71, led to the construction of a stream stripper to treat the toxic, foul condensates.

Domtar is presently exploring a multitude of in-plant treatment systems, most of which will result in a reduction of toxicity. Some treatments might include high pH lime treatment, re-utilization of various waste water streams, dry barking, improved recovery systems, and other engineering designs. It is our belief that in-plant treatment systems are more compatible with process design and will alleviate the necessity for energy consuming biological treatment.

In the summer 1975, as part of a larger program, the Domtar Research Centre determined the relative toxicity of a number of process streams. Because of the variability of effluent quality in the process streams of a pulp and paper mill, it was necessary for the streams to be sampled and bioassayed simultaneously. Due to the large numbers of tests to be run in a limited space, it was necessary to utilize an organism other than fish. Macro-invertebrates commonly found in lakes, rivers and streams are an important link in the aquatic food chain. The Daphnia (water flea), is easily reared in the laboratory and because of its small size could easily be accommodated by the practical limitations outlined above.

In the 1940's and 50's considerable insecticide research was conducted with Daphnia (1-5). As a result, numerous culturing and bioassaying methods have been developed. More recently, the government of France adopted Daphnia as a standard bioassay animal for the evaluation of their municipal waste water. Biologists at MacMillan Bloedel Limited in British Columbia are presently investigating, with reported success, the use of Daphnia bioassays to assess the toxicity of pulp and paper wastes (personal communications). The petroleum industry in the United States is also investigating the usefulness of Daphnia bioassays. It should be noted that the utilization of Daphnia in this study was not intended to replace rainbow trout as the standard bioassay animal, but rather to use as an alternate test animal in situations where the use of fish bioassays was restrictive.

Materials and Methods

Fish Rearing

Rainbow trout weighing 0.5 to 2 gm each were used in the fish bioassays. Fish were held in unchlorinated well water with a flow rate of approximately 1.4 l of water/gm. of fish/day (10). The water was first passed through a sand filter and then into a temperature regulation reservoir. Temperature was regulated by means of a 1 H.P. refrigeration unit. The water was allowed to overflow from the reservoir to the rearing tanks. This arrangement produced a dissolved oxygen concentration of 8-10 mg/l. In order to minimize the growth rate of the fish the water temperature was maintained at 10-11°C.

Fish were acclimated to the well water for two weeks before being used in a bioassay. However, this period of acclimation may not always be possible, when conducting on-site bioassays.

The fish, fed a dried commercial fish chow, were exposed to a 12-hour dark/12-hour light photoperiod.

Fish Bioassays

The static bioassays were conducted in plastic pails with plastic liners. The volume of the test solution varied with the size of fish. A loading density of one gram of fish per one litre of solution was followed. Before an effluent was diluted for testing, the pH was measured and adjusted to pH 7 with HCl or NaOH, if it was outside the pH 6-9 range Four concentrations, usually 25, 50, 75, 100%, unless an effluent was known to be very toxic (10), were then prepared for each effluent tested. Ten fish were placed in each concentration. Mortality checks and removal of dead fish, if any, were made frequently on the first day of the test (0.25, 0.5, 1, 2, 4, 8 hr) and then once a day for the remainder of the test (four days or 96 hr). During the test, pH, temperature, (10°C ± 1°C) and dissolved oxygen were monitored. When dissolved oxygen was less than 9 mg/l, pure oxygen, rather than compressed air, was bubbled into the test solutions thus reducing the stripping of volatile compounds by aeration.

Daphnia Rearing

The original Daphnia stock (a mixture of D. magna and D. pulex) was obtained from Dr. Rob Peters of McGill University. The two species were not separated for use in these tests.

Initially, a variety of culture media were evaluated. Typical of these were:

(1) A boiled lettuce leaf placed in five gallons of unchlorinated well water. The lettuce provided a substrate for bacteria to grow which in turn were consumed by the Daphnia. This was occasionally supplemented with a few drops of yeast. (6)

(2) A manure-soil infusion made from five grams of manure mixed with 25 g of dried sandy muck in one litre of water. This mixture was allowed to stand two to three days, filtered, and then allowed to stand another four to six days before the filtrate was used as a medium. (5)

(3) An algae culture was originally started by placing goldfish in a 10 gal. aquarium. There was sufficient algae in the gastrointestinal tract of the goldfish to serve as inoculum for a healthy growth of algae in the aquarium. The presence of the goldfish and their waste products provided a continuous stock of algae. This solution of algae is sometimes referred to as "green water".

(4) Powdered, dry Purina trout chow.

The medium which produced the best growth and reproduction in Daphnia included equal parts of unchlorinated well water, the manure-soil infusion and green water mixed in a ten gallon aquarium. Once the stock was established in this medium, the addition of 250 ml of green water every two days to a ten litre aquarium of Daphnia was sufficient to maintain a healthy, reproducing stock. Occasionally, water was added to compensate for evaporation losses in the aquarium. The culture was not aerated since sufficient oxygen was supplied by the algae and natural diffusion. A 16-hour light/8 hour dark photo period was maintained. The rearing and bioassay of the Daphnia were conducted at ambient temperature without temperature control.

Daphnia Bioassays

Daphnia bioassays were carried out in 30 ml beakers with a test volume of 25 ml. Four dilutions were prepared for each stream tested in the same manner as the fish bioassays and ten Daphnia were carefully pipetted into each beaker. Particular care was taken when transferring the animals so that they would not become trapped in the surface film of the test material.

The pH of the effluent was checked and adjusted if it fell outside the range of 6-9 (as in the fish bioassay) before the Daphnia were transferred to the test vessel. The effluents were not aerated during the test, since all of the oxygen required was provided by diffusion at the surface of the test solution. Oxygen measurements were recorded for each effluent prior to the bioassay. However, measurements were not taken at the end of the bioassay.

The duration of the Daphnia bioassays was 24 hours. Personal communications with biologists at MacMillan Bloedel, the literature and a few preliminary experiments by our own lab indicated that this length of test could possibly provide results equivalent to the four day (96 hr) trout bioassay. The Daphnia bioassays were compared to 24 and 96 hr static trout bioassays.

Results and Discussion

The primary objective of the study was to evaluate various in-plant streams in a pulp and paper mill for their toxic contribution to the total mill's toxicity. To do this representatively, it was necessary to sample and test several streams at the same time.

In this study, several streams (up to 20) were sampled simultaneously. All of these streams were bioassayed with Daphnia while a maximum of nine were tested with fish. The process streams were sampled and bioassayed on four separate days.

The results (24 hr LC_{50}'s) of the Daphnia bioassays are summarized in Table 1. As can be seen, many of the LC_{50} results obtained on the four bioassay days were similar to one another. When the streams were toxic, a greater variability in LC_{50} values were obtained from one day to another. This might be accounted for by the daily process fluctuations encountered in a complicated procedure such as pulping.

TABLE 1

DAPHNIA TOXICITY BIOASSAY RESULTS

Percent solution (v/v) resulting in death of
50% of test animals in 24 hr. (24 LC_{50})

Stream	Series 1 (20 Aug 75)	Series 2 (25 Aug 75)	Series 3 (28 Aug 75)	Series 4 (3 Sept 75)
Total mill effluent	N.L.	>75	90*	N.L.
Clarifier	N.L.	67	N.L.	N.L.
Low solids stream	N.L.	N.L.	N.L.	N.L.
Kraft mill sewer	80*	N.L.	N.L.	N.L.
#2 Paper Machine	N.L.	N.L.	70*	N.L.
#2 Paper Machine filt.	N.L.	N.L.	>75	N.L.
Groundwood clean	-	N.L.	N.L.	N.L.
#1 Paper Machine	N.L.	N.L.	-	N.L.
Bleach Plant:				
Chlorination	75	N.L.	80*	N.L.
Caustic + Hypo	86	86	78	61
Kiln + Stripper Overflow	N.L.	N.L.	-	43
Causticizing + kiln	N.L.	70	56	25
Groundwood ww	61	61	31.5	50
Woodroom	<25	>5	53	>50
#2 Evaporator	<25	N.L.	<25	<25
#1 Recovery	-	<25	N.L.	>50
#2 Recovery	-	N.L.	<25	<25
Stripped feed	1.7	>5	-	5.4
Stripper effluent	<25	<25	-	stripper down

Non Lethal (N.L.)-100% survival of the test animals after 24 hr.
exposure at 100% conc. of the test solution.

* These figures represent the percent survival in 100% concentration of the test solution after 24 hr.

Because of this fluctuation in toxicity of certain process streams, it was difficult to predict a range of dilutions which would give a maximum number of partial responses. This was especially critical with Daphnia bioassays since the difference in effluent concentration at which they all survived or all died during the test was quite narrow.

The results of both the Daphnia and fish bioassays are summarized in Table 2. The fish bioassays are reported as 24 and 96 hour LC_{50}'s.

Daphnia were not as sensitive to the pulp and paper effluents as were the fish. In the majority of the streams tested with both animals, more Daphnia bioassays showed no toxicity. However, in the more toxic streams (woodroom, groundwood white water and stripper feed & effluent) the Daphnia bioassays usually showed a greater tolerance than the fish. It is important to realize that Daphnia did not, under our test conditions, respond to the effluent in a similar fashion to rainbow trout. Nevertheless Daphnia is an extremely useful bioassay tool since it is able to indicate with a minimum of effort which streams are toxic or non-toxic and can rapidly focus on the very toxic process streams.

Although this preliminary study with Daphnia showed no direct correlation between the trout and the Daphnia bioassay, it did show that the Daphnia bioassay pointed out areas of extreme toxicity. The main objective in using the Daphnia bioassay, especially on-site, was to simultaneously test all the effluent streams in the mill thereby pinpointing the "hot spots" or extremely toxic streams. These streams would then be more closely monitored with the trout bioassay since these streams would be the most likely candidates for in-plant treatment systems.

TABLE 2

COMPARISON OF FISH AND DAPHNIA BIOASSAY RESULTS

Stream	Fish Bioassay		Daphnia Bioassay
	24 hr LC_{50}	96 hr LC_{50}	24 hr LC_{50}
Total mill effl.	35.0	28.0	N.L.
	>75.0	46.5	>75.0
	>75.0	60.0	N.L.
Clarifier	35.0	22.5	N.L.
	>75.0	>75.0	67.0
	94.0	72.0	N.L.
Low solids stream	65.0	55.5	N.L.
	35.0	26.0	N.L.
	38.5	<25.0	N.L.
Kraft mill sewer	16.0	15.5	80*
	>50.0	>50.0	N.L.
	>75.0	37.0	N.L.
#2 Paper Machine	90*	70*	N.L.
	74.0	64.0	N.L.
	N.L.	N.L.	N.L.
Woodroom	>5.0	5.0	<25.0
	>10.0	>10.0	>5.0
Groundwood ww	20.0	<20.0	61.0
	-	-	50.0
Stripper-feed	1.7	1.7	1.7
	>5.0	>5.0	>5.0
	3.8	3.8	5.4
Stripper effluent	16.0	9.1	<25.0
	17.0	17.0	>25.0

Non Lethal - 100% survival of the test animals after 24 hr. exposure at 100% conc. of the test solution.

* These figures represent the percent survival in 100% concentration of the test solution after 24 hr.

There are several possible explanations for the differences observed between the Daphnia and the trout bioassays.

(1) The stock of Rainbow trout might have been weakened in transport and were therefore more susceptible to the stress factors placed on the fish by the effluent. This point strongly reinforces the necessity of conducting a reference toxicant test on each stock of fish and Daphnia. Unfortunately, in this study, a reference toxicant test was not conducted due to lack of time and space.

(2) The surface area to volume ratio in the Daphnia bioassays was approximately ten times greater than that of the fish bioassays. Therefore, the actual amount of toxic material in the Daphnia bioassays may have been less than that of the fish due to natural volatilization of components released at the surface. This problem could be easily eliminated by using test tubes instead of beakers so that the surface area is reduced in the Daphnia bioassay.

(3) At lower temperatures, the body metabolism of the Daphnia is lowered. This results in a greater tolerance to toxic compounds. In this study, the Daphnia were exposed to a broad temperature range (6° - 15°C) since the test was conducted at ambient temperature which varied throughout the day. However, the trout were kept at a more or less constant temperature of 14°± 1°C.

CONCLUSIONS

This summer's initial on-site use of the Daphnia bioassays dramatically pointed out the many advantages of using a smaller bioassay animal in conjunction with the standard trout bioassay.

(1) The size of the Daphnia significantly reduces the amount of equipment, space, and volumes of effluent necessary in conducting a bioassay.

(2) Numerous streams may be sampled and tested at one time.

(3) A healthy stock of Daphnia is easy to maintain and transport in the case of on-site testing.

(4) Aeration is felt to be unnecessary in a Daphnia bioassay and, therefore, the problem of stripping-off by aeration of volatile compounds present in the effluent is eliminated.

(5) The test period is shorter, therefore there is a rapid feedback of results.

(6) The time required to set up a Daphnia bioassay is half that of a trout bioassay.

(7) Daphnia are less susceptible to stresses (6) and are therefore easier to transport to a mill site.

(8) Because of the simplicity of the Daphnia bioassay, on-site assaying by mill personnel would become possible.

Although this preliminary study with Daphnia showed no direct correlation between the trout and the Daphnia bioassay, it did show that the Daphnia bioassay pointed out areas of extreme toxicity. The main objective in using the Daphnia bioassay, especially on-site, was to simultaneously test all the effluent streams in the mill thereby pinpointing the "hot spots" or extremely toxic streams. These streams would then be more closely monitored with the trout bioassay since these streams would be the most likely candidates for in-plant treatment systems.

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DISCUSSION OF STANDARD BIOASSAY METHODOLOGY

Ed Pessah

One of the things I have often wondered about is the effect of maintaining fish stocks at low temperatures, boosting them up to 15°C, then using them in a bioassay test. We found that, with pumpkinseed sunfish, a 5°C shift produced a growth surge which lasted about twelve weeks. If this change is metabolically related and if the toxicant affects metabolism, I wonder if a rapid temperature change would give you a different result?

Gary Vikers

Mr. Thackery, in your comparison of costs on raising laboratory fish versus hatchery fish, I did not see any account for manpower. What would that cost be?

Tom Thackery

We figure the cost would be about $300 for manpower, that would include counting the eggs, sorting them and removing any spoiled eggs.

Don McLeay

Mr. Thackery, B.C. Research was comparing methods of rearing rainbow trout fry versus incubating eggs for routine use in bioassays. We are now coming to the conclusion that it is more economical to purchase swim-up fry at monthly intervals as long as the source is available. We have now managed to locate a source in the United States that can provide swim-up fry for eight to ten months of the year. These fry can be delivered by plane and there is no cost incurred in picking and maintaining the eggs. We also find that for our studies on extracted toxicants produced from pulp mill effluents we like to use very small fish because we are limited by the amount of toxicant available. The size of fish that we use are as small as 0.2 to 0.3 grams and we will not use any fish until they are feeding actively. We have run some reference toxicant tests with very small fish versus large fish and found no difference. Therefore with industrial wastes which are low in toxicity, there is a marked advantage in using very small test fish. Do you find that fish size makes any difference? Have you tested this?

Tom Thackeray

We found that fish size does not make a difference in response to the chemicals used, however, the size disparities were not large. The hatchery fish were between 3 and 3.5 grams but the laboratory fish were usually less than 3 grams.

Gary Alexander

Mr. Wells, in your comparison of the fathead minnows and the rainbow trout in the static bioassays, was the loading density the same in all three cases?

Dave Wells

The loading rates would be comparable.

Gary Alexander

Mr. Wells, you commented that obviously acute lethal levels of well known toxicants such as cyanide are too obvious to necessitate a bioassay. How could you account for levels of heavy metals that are complexed and not in a toxic form.

Dave Wells

You can never second guess a trade waste and its toxicity. If a waste is tested knowing it contains high concentration of a recognized toxicant, hindsight dictates that there was not much point in running a bioassay. However, it is now our practice to bioassay all trade wastes received in our laboratory, at least once, in order to allow the fish to provide the final integration of toxicity.

John Toby

Mr. Wells, have comparisons been completed between freshly run bioassay samples and those that have been stored in a refrigerator.

Dave Wells

Not to my knowledge. The best I can say is that there is not a consistent response by the fish. Some effluents appear to lose toxicity when they are stored, other samples appear to maintain their toxicity but it does not appear to be a consistent factor. The best representation of an effluent would be to collect a sample and run a bioassay on it as soon as possible and preferably refrigerate between collection time and establishment of the bioassay.

Gerard LeDuc

Miss Naish, at what temperature did you run the bioassays compared to the Daphnia culture?

Miss Naish

The Daphnia bioassays were carried out at 10-15°C. The culture was held at 23°C.

Gerard LeDuc

John Davis, concerning temperature, I think there is an obvious lack of regard to cold temperatures in bioassays. Too often we are working at room temperature and if you look at the various water quality criteria handbooks most of the bioassay results or LC-50's are reported for 18-22°C. Water temperatures in the winter and in Canada's North rarely exceed 10-12°C and I think too often we assume that cold temperatures have less of an effect than warm temperatures. We have found that Daphnia are more sensitive to arsenic at low temperatures than what is referred to in the literature at temperatures of 18-22°C. I think that reference toxicants and trade wastes bioassays should include lower temperatures.

Jack Klaverkamp

At the Freshwater Institute we heard a seminar by Dr. Hansen from the University of Hamburg. He was using Daphnia in a food chain experiment and he passed along some interesting notes of caution concerning diet. He was comparing the lipid content in Daphnia that were captured in the field versus those that were raised in the laboratory and measuring the Lindane content in the lipid. First of all he found a five-fold difference in lipid content between laboratory reared Daphnia and those in the field. The laboratory Daphnia contained more lipid and consequently were more resistant to Lindane because of the greater lipid pool. Perhaps the Daphnia could be starved a day or so before to reduce lipid content.

David Wells and John Davis, it seems that about two years ago there was a certain amount of emphasis on rigorously standardizing bioassays and one of the aims of this was to provide a better basis of comparison in legal cases. I feel that this has drifted by the wayside in the last couple of years and would like to know how you feel about this. Would you comment on the legal use of bioassays.

John Davis

I think that probably you are right in that there has been a tendency to move away from this rigidity. Regulatory agencies have been accepting the best practicable biological technology for effluent treatment. However, I feel that we will still see these agencies requiring a rigid test with very definite guidelines that will be used and defended in court.

Dave Wells

We have been involved in a number of court actions involving the use of bioassays, and the attitude is that the results of the Ministry are inviolate and not open to challenge. What we would prefer to be known is that we do try to run a standardized bioassay with all the best practices.

Ed Pessah

I think you can go into court with just about anything as long as it holds water. We have proposed a standard bioassay test in order to develop, not so much a legalistic tool, but a uniform standard for industry. People in B.C. have been going to court with static bioassays and winning cases and so have we on the East coast. The static bioassay is what I consider a first level of protection. What we are suggesting is that the equivalent to the 96-hour flow through bioassay is that first level.

The other thing that I would like to comment on is the usefulness of that kind of test. It gives us a single point of focus. It is difficult enough to go through the Water Quality Criteria Handbook (EPA, 1973) trying to figure out what is toxic to different species under different conditions. I think there is a definite advantage to having at least one specific test that can provide a common reference point.

TOPIC II

REFERENCE TOXICANTS

An Evaluation of Phenol and Sodium Azide as Reference Toxicants in Rainbow Trout

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The use of reference toxicants to improve the quality of toxicity testing procedures has been discussed recently by Davis and Hoos (1975). In general, these toxicants facilitate interlaboratory comparisons of toxicity data and assist in standardizing bioassay procedures. The purpose of this report is to present data which evaluate phenol and sodium azide for use as reference toxicants.

Table 1 presents the six criteria which were chosen for this evaluation. First, the chemical should be toxic in concentrations at or below the low mg/l range. Second, analytical techniques which are

TABLE 1

Criteria for evaluating chemicals as reference toxicants:

1. Toxic at low concentrations
2. Simple analysis of the chemical in water
3. Available in a purified grade
4. Very soluble in water
5. pKa > pH + 1 or pKa < pH - 1
6. Background information on the modes and sites of action.

TABLE 2

Water characteristics - Winnipeg City Tap

	mg/L
Chloride	5.0 ± 0.7
Sulfate	7.1 ± 0.7
Sodium	1.7 ± 0.2
Potassium	1.4 ± 0.1
Magnesium	5.1 ± 0.1
Calcium	22.1 ± 0.5

Conductivity = 165 ± 6 μmho/cm^2
Hardness (Ca, Mg) = 76

($\overline{X}$ ± S.D.; n = 12)

relatively simple, should be available to determine the actual concentration of toxicant in the water. Third, the chemical should be easily available in a purified grade. Fourth, the chemical should be very soluble in water to avoid the use of emulsifying, solubilizing, or suspending agents. Fifth, if the chemical is an organic acid or base, the negative log of the dissociation constant (the pKa) should be at least one unit from the pH of the water. This would ensure that at least 90% of the chemical would be in the ionized or unionized form. Finally, if background information on the mode and sites of action of the toxicant is known, abnormal responses of fish would be more readily apparent.

Preliminary investigations in our laboratory indicated that phenol and sodium azide met these criteria and therefore they should be evaluated as reference toxicant candidates. To conduct acute lethality experiments, a Mount and Brungs (1967) proportional dilutor system modified as described by Harrison et al (1975) was used to deliver 5 concentrations in duplicate or 10 single concentrations of toxicant. Experimental parameters of the acute lethality experiments using 5 concentrations in duplicate include: temperature: 15°C; aeration: continuous with airstone; no. of fish per concentration: 20; no. of fish per vessel: 10; vessel volume: 20 liters; flow rate to each vessel: 60 ml/min.; 95% of replacement time: 17 hours; and times of mortality checks (hours): 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4, 5, 6, 7, 13, 24, 48, 72 and 96. For the ten single concentrations of toxicant these parameters were the same except the no. of fish per concentration was 10.

Table 2 presents some water chemistry data on anions, cations, conductivity and hardness. Each value represents the mean and S.D. of twelve analyses.

Table 3 presents the LC_{50} results of four phenol bioassays and a summary of the test conditions. The five phenol test concentrations used in each bioassay were in 50% proportional ranges, viz. 50 mg/L, 25 mg/L, 12.5 mg/L etc. In a given concentration of phenol, it was often observed that no fish would be dead after 24 or 96 hours, but in the next highest concentration all, or almost all, of the fish would be dead. Consequently, the straight-line graphical interpolation method (Standard Methods, 1971) of estimating the LC_{50} was used, rather than log-probit methods (Litchfield and Wilcoxon, 1949: Finney, 1971).

If trout could survive for 24 hours in a given concentration of phenol, they usually survived for the 96 hour duration of the bioassay. Therefore, in a given experiment there is no or very little difference in the 24 hour and the 96 hour LC_{50} value. Similar observations with phenol have been made by Lammering and Burbank (1960) in the bluegill sunfish and by Brown, Shurben and Fawell (1967) in the rainbow trout.

The use of log-probit methods can provide a more accurate LC_{50} value. However, these methods require the use of toxicant concentrations which produce mortality only in part of the test population in at least two concentrations. Therefore a bioassay was conducted using 10 concentrations of phenol in a 75% proportional range. Figure 1 presents the results of this bioassay.

Phenol concentration and median survival time (MST) have been plotted logarithmically as recommended by Sprague (1969) and Brown (1973). The vertical lines represent the 95% confidence limits as determined by Litchfield (1949). Note that the relationship between concentration and MST is curvilinear with a very steep slope. There were only two mortali-

TABLE 3

PHENOL

Experiment	LC_{50}(mg/L) 24h	96h	Loading density(g/L)	Temp(°C)	pH	DO(mg/L)	Wt(g)	L(cm)	C.F. (Wt/L^3 x 1000)
1	7.7	7.7	2.37	15.1±0.1	7.9±0.1	8.7±0.5	4.74±1.44	7.14±0.73	1.30
2	10.0	10.0	2.17	15.1±0.1	7.9±0.1	8.7±0.5	4.34±1.11	7.08±0.67	1.22
3	10.2	9.1	2.67	14.8±0.6	7.9±0.1	9.3±0.4	5.34±1.30	7.60±0.58	1.22
4	8.9	8.5	2.63	14.8±0.6	7.9±0.1	9.3±0.4	5.27±1.70	7.52±0.84	1.24

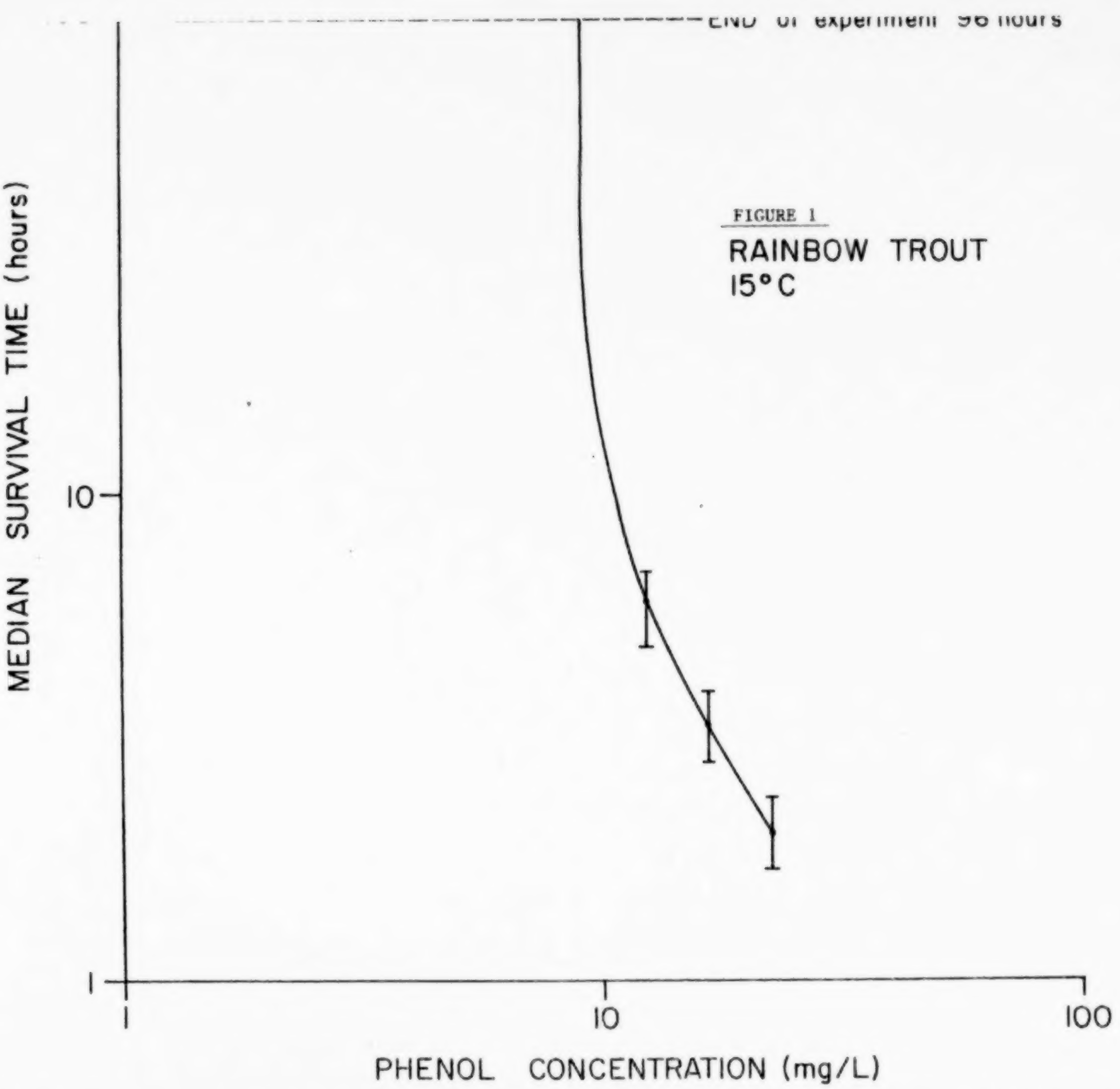


FIGURE 1

RAINBOW TROUT
15°C

ties in the 8.3 mg/L concentration at 96 hours, whereas the MST in the 12.3 mg/L concentration was 5.8 hours. Curvilinear toxicity curves have also been shown for cyanide and ammonia (Sprague, 1969(.

Table 4 presents the LC_{50} results of three azide bioassays and a summary of the test conditions. The five azide test concentrations used in each bioassay were in 75% proportional ranges, viz. 10 mg/L, 7.5 mg/L, 5.625 etc. With the exception of the 24 hour value in Run #3, an adequate number of data points were obtained in these bioassays to use the log-probit method of Finney to calculate the LC_{50} values. The 24 hour value in Run #3 has been calculated by the straight-line graphical interpolation method. Please note that there is an approximate 3 fold difference between the 24 hour LC_{50} and the 96 h. LC_{50}.

Figure 2 presents the results of a bioassay, which was conducted using 10 concentrations of azide in a 75% proportional range. Note that the relationship between concentration and MST is straight line with a gradual slope. Straight line toxicity curves with a gradual slope have also been observed for copper and zinc (Sprague, 1969).

The chemicals used for reference toxicants, as with any other type of toxicant, have absorption or adsorption affinities for the various neoprene, polyethylene, tygon, glass, etc. components of bioassay test vessels and dilutor systems. Therefore, a significant difference could occur between the calculated concentration and the actual concentration of chemical in the bioassay test vessel water. Consequently, there should be available relatively simple chemical analyses to determine the actual concentration of reference toxicant in water. Relatively simple procedures include colorimetric methods using a spectrophotometer, but do not include chromatographic methods e.g. gas-liquid chromatography.

TABLE 4
AZIDE

Experiment	LC_{50}(mg/L) 24h	96h	Loading density(g/L)	Temp(°C)	pH	DO(mg/L)	Wt(g)	L(cm)	C.F. (Wt/L^3 x 1000)
1	10.52	2.75	2.38	14.9±0.1	7.7±0.1	9.6±0.3	4.76±1.36	7.32±0.68	1.21
2	7.09	2.84	3.04	14.8±0.2	7.7±0.1	9.5±0.4	6.07±1.96	7.87±0.84	1.25
3	(9.60)	3.28	3.92	14.4±1.5	7.7±0.2	9.3±0.8	7.84±3.41	8.57±1.12	1.24

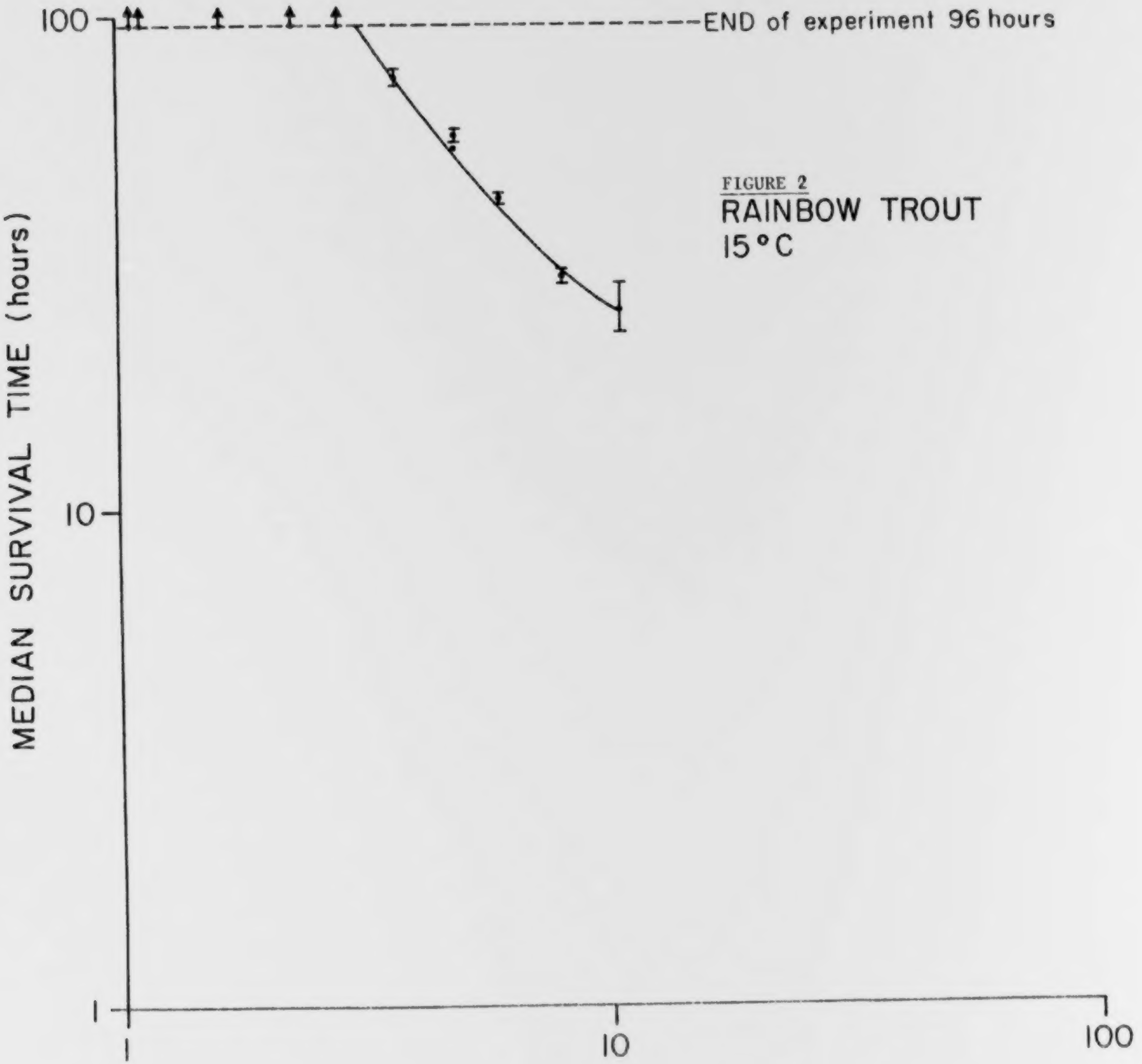


FIGURE 2
RAINBOW TROUT
15 °C

Figure 3 presents data on the actual concentrations of phenol, expressed as the per cent of calculated value. Phenol concentrations were determined colorimetrically by using the antipyrine dye method described in Standard Methods (1971). Numbers in parentheses represent the number of water samples taken from four separate acute lethality experiments. These data, which are expressed as the mean ± S.D., have been grouped according to concentration ranges and sampling times. The lowest concentration was 75% of expected or calculated in the 0.5 mg/L to 9.0 mg/L range at 96 hours; the highest was 99% of expected or calculated in the 10 mg/L to 29 mg/L range at the 0-12 hour interval.

Azide concentrations in water were determined by reacting azide with a known amount of added nitrite to form nitrous oxide and nitrogen. The unreacted nitrite was converted to a pink azo dye compound by reacting with sulfanilic acid and N-1-naphthyethylene diamine. After these reagents had been allowed to react for 90 minutes, absorbance was measured at 540 nm. This analysis is not specific for azide, since the presence of any nitrite-consuming material, e.g. halogenates, hydrazine salts, or hydrogen peroxide, will influence the formation of the azo dye.

Figure 4 presents data, expressed as the per cent of calculated value, on the actual concentrations of azide. Numbers in parentheses represent the number of water samples taken from four separate acute lethality experiments. These data, expressed as the mean and standard deviation, have been grouped according to concentration ranges and sampling times. Note that all of the values are over 100% of the calculated values, but fall in the range of 106% (4.0 to 10.0 mg/L at the 13-72 hour interval and at 96 h.) to 116% (0.1 to 2.4 mg/L at 96 hours). These slightly high values are probably due to the non-specific nature of the azide analysis

FIGURE 3

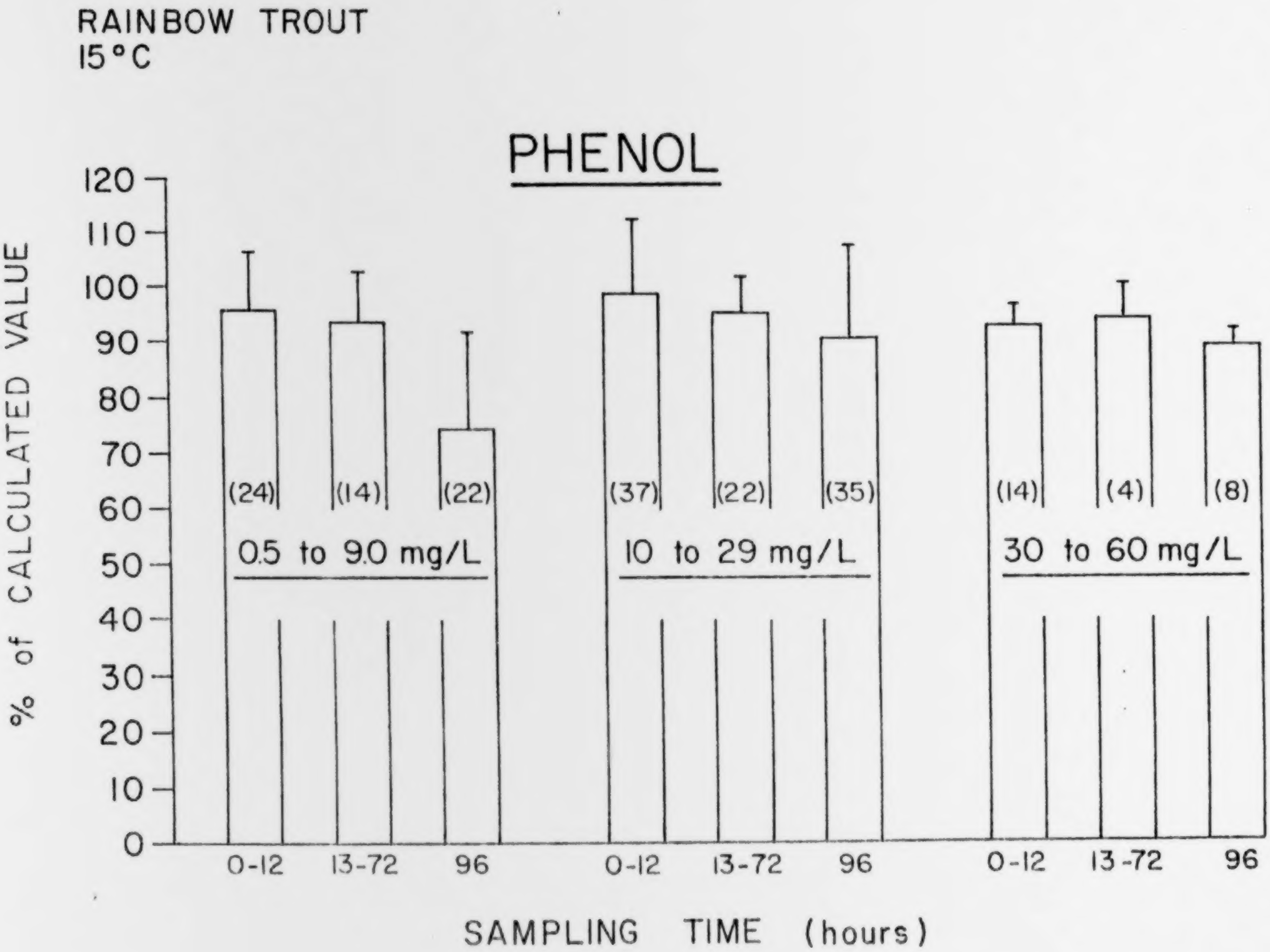
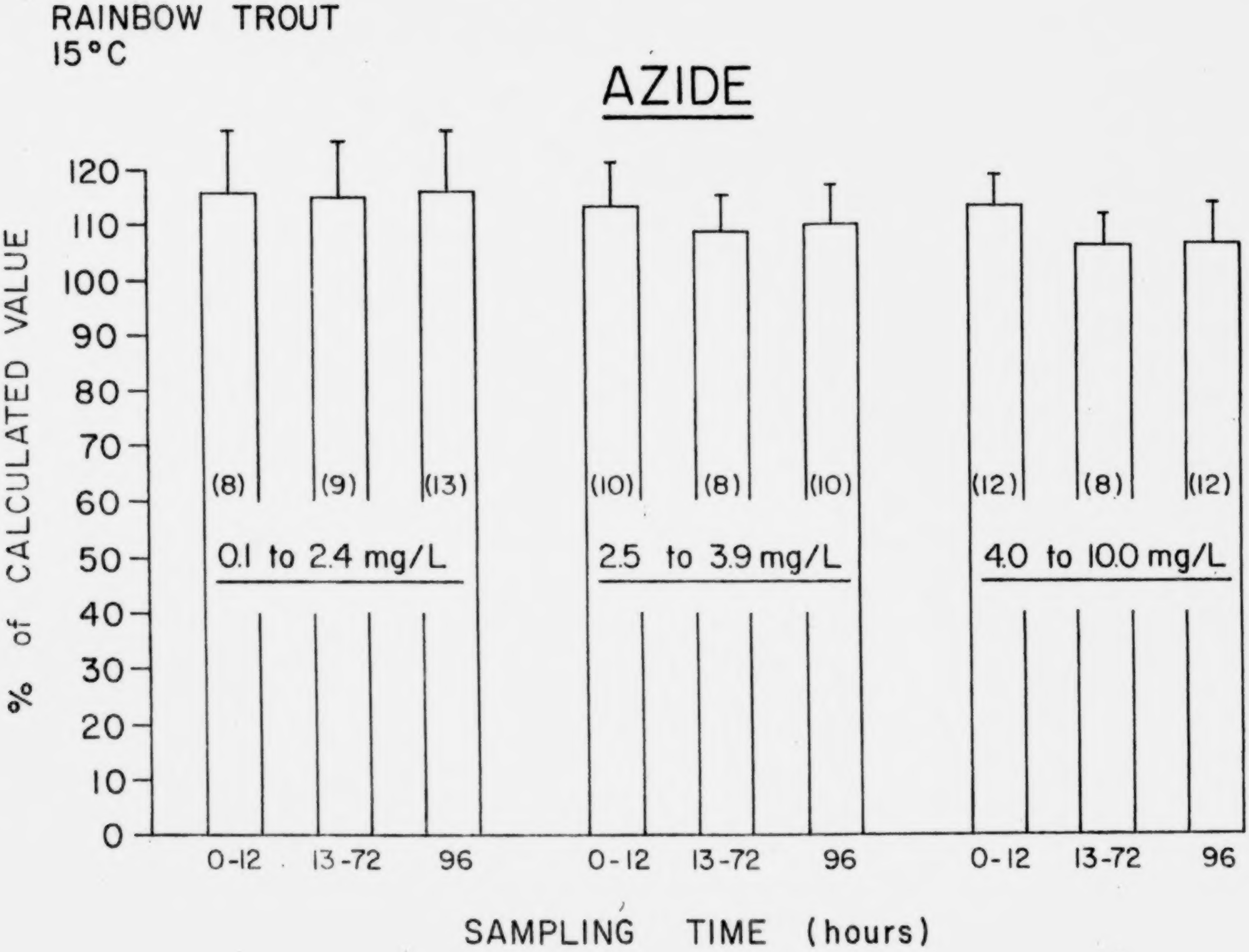

RAINBOW TROUT
15°C
PHENOL
% of CALCULATED VALUE
0
10
20
30
40
50
60
70
80
90
100
110
120
(24)
(14)
(22)
(37)
(22)
(35)
(14)
(4)
(8)
0.5 to 9.0 mg/L
10 to 29 mg/L
30 to 60 mg/L
0-12
13-72
96
0-12
13-72
96
0-12
13-72
96
SAMPLING TIME (hours)

FIGURE 4


RAINBOW TROUT
15°C
AZIDE
% of CALCULATED VALUE
120
110
100
90
80
70
60
50
40
30
20
10
0
(8)
(9)
(13)
(10)
(8)
(10)
(12)
(8)
(12)
0.1 to 2.4 mg/L
2.5 to 3.9 mg/L
4.0 to 10.0 mg/L
0-12
13-72
96
0-12
13-72
96
0-12
13-72
96
SAMPLING TIME (hours)

described above.

Interest in the use of reference toxicants for inter-laboratory comparisons of toxicity data extends across Canada and the United States. Consequently these chemicals should be readily and easily available in a purified form throughout these countries. The availability of phenol and sodium azide from six international chemical companies is presented in Table 5. Phenol is available in a recrystallized form of 99% or greater purity from all six suppliers. Sodium azide is available in a purified or technical grade from five of the six companies.

To avoid the use of emulsifying, solubilizing, or suspending agents and to minimize absorption, reference toxicants should be very soluble in water. Table 6 presents data on the water solubility of phenol and sodium azide. Phenol is soluble in water to the extent that a concentrated solution of about 6.6% W/V can be made. This solution, which is the equivalent of 66,000 mg/L, is approximately 6 times the concentration required to make the stock solution for phenol bioassays in our system. Sodium azide is even more water soluble than phenol, since concentrated solutions of approximately 40% W/V can be made.

Organic acids and organic bases are characterized by a pKa value, which can be defined as the pH at which 50% of the total amount of chemical is un-ionized and 50% is ionized. Since it is usually the un-ionized species of chemical, which diffuses through cellular membranes, such as the gills, the pH of the water and the pKa of the chemical are very important in determining the amount of chemical transported from the water into the fish. Consequently, our fifth criteria states that organic acids and bases used as reference toxicants should have pKa values which are at least

TABLE 5

AVAILABLE IN A PURIFIED GRADE

	Phenol	Sodium Azide
Aldrich	Yes	No
J.T. Baker	Yes	Yes*
BDH	Yes	Yes
Eastman	Yes	Yes*
Fisher	Yes	Yes
Matheson Coleman & Bell	Yes	Yes

*Practical grade

TABLE 6

VERY SOLUBLE IN WATER

Source	Phenol	Sodium Azide
Handbook of Chemistry and Physics, 55th edition	soluble; soluble in all proportions	41.7 g/100 ml cold water
Merch Index, 8th edition	1 g in about 15 ml	40.2g/100 ml at 10°C; 41.7 g/100 ml at 17°C

one unit from the pH of water. Table 7 illustrates why this is important. The pKa of phenol, a weak organic acid, is about 9.8 at 15°C. Consequently, at pH 9.8, 50% of the phenol is un-ionized and 50% is ionized. From the Henderson-Hasselbalch equation the ratio of un-ionized to ionized chemical at each pH value can be calculated. Since the pH of the water used in the phenol bioassays was 7.9, almost 99% of the chemical was in the un-ionized form and small variations in pH would not significantly influence the amount of un-ionized phenol. Although pH can influence the solubility and stability of inorganic salts, such as sodium azide, the amount of chemical in solution is 100% ionized at all pH values.

The sixth criterion for the evaluation of reference toxicants concerns the existence of background information on the sites and mechanisms of action. It is thought that the lethality produced by phenol in fish is caused by adverse effects on gill and nervous sytems. Phenol also produces toxicity in a non-specific manner on blood, neuromuscular, liver, spleen and cardiovascular systems. Although reports on azide toxicity in fish are not as plentiful as those on phenol, considerable work in mammalian and bacterial systems has shown that it inhibits cellular respiration in a manner identical to cyanide. Studies measuring serum electrolytes and tissue glycogen content, which may be useful in understanding the mechanism of action of azide toxicity in rainbow trout, have been initiated.

From these studies we conclude that phenol and sodium azide meet the six criteria. Interesting differences exist between the two chemicals and it may be desirable to use both as reference toxicants. For example, phenol is characterized by a curvilinear, steep-slope toxicity curve, whereas a straight-line, gradual slope curve was obtained for azide. There are also large differences in the degree of ionization between each chemical at neutral

TABLE 7

pKa > pH + 1 or pKa < pH - 1

pH	Phenol	Sodium Azide
11.8	99% ionized	-a salt therefore ionized at all pH values
10.8	90% "	
9.8	50% un-ionized	
8.8	90% "	
7.8	99% "	
6.8	99.9% "	
5.8	99.99% "	

pH values.

We recommend the incorporation of phenol and sodium azide in testing programs which are designed to select reference toxicants.

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DODECYL SODIUM SULPHATE (DSS) AS AN INTRALABORATORY REFERENCE TOXICANT IN FISH BIOASSAYS

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ABSTRACT

Dodecyl sodium sulphate (DSS) has been used for two years as an intralaboratory reference toxicant to quantify the relative health and sensitivity of fingerling rainbow trout, *Salmo Gairdneri*, used for acute lethal bioassays.

The mean 4 day LC50's for DSS varied from 4.5 to 6.2 mg/l and were influenced by both mode of bioassay and size of fish. DSS seemed capable of measuring changes in sensitivity of fish used for oil-plus-oil dispersant mixtures. No change in toxicity in DSS was recorded for fish just transported from hatchery to laboratory or when mortality occurred in the holding stock of fish.

It was concluded that DSS is useful as a reference toxicant when testing chemical systems such as oil-plus-oil dispersant mixtures. DSS is not considered to be a useful indicator of the general health and sensitivity of fish stocks if transportation results in stress of fish and if mortality in holding tanks is indicative of lower general health of fish.

The need for reference test systems to provide information prior to carrying out definitive bioassays and the potential usefulness of test systems using various responses to depict the general health and sensitivity of test fish is discussed.

1. INTRODUCTION

Interest is often expressed in standardizing bioassay procedures from laboratory to laboratory within Environment Canada. There have been several reports on the use of reference toxicants during bioassays (Alderdice, 1963; Marking, 1966; LaRoche *et al*, 1970; Anon, 1973), the most recent and comprehensive being that of Davis and Hoos (1975). The latter authors reported the use of two reference toxicants as a laboratory intercalibration exercise and found that their use provided "useful information on the comparability of fish toxicity tests by independent laboratories".

This paper describes the function of reference toxicants, reviews the chemistry and toxic action of a reference toxicant, dodecyl sodium sulphate (DSS), and presents data from this laboratory on the use of DSS as an intralaboratory reference toxicant.

2. FUNCTION, CHEMISTRY AND TOXIC ACTION

2.1 Function of a Reference Toxicant

1) To measure the health and sensitivity of test organisms (LaRoche *et al*, 1970; Davis and Hoos, 1975). This function provides a means of detecting large differences in sensitivity of fish stocks to toxicants that may be the result of health of the stock or some variation in bioassay conditions (Davis and Hoos, 1975).

2) To establish and define standard testing conditions, essential for obtaining estimates of comparative toxicity in the pharmacological sense (Alderdice, 1963). Implied here is the standardization of conditions relating to the water used in particular test situations (Doudoroff *et al*, 1951).

3) To compare the relative toxicities of substances by using the toxicant as an internal standard (Hart, Doudoroff and Greenbank, 1945; LaRoche *et al*, 1970).

4) To link the work of different investigators (Hart, Doudoroff and Greenbank, 1945; LaRoche et al, 1970). However, results of independent bioassays often cannot be related because the comparative resistance of many different species of test fishes has not been established (Douglas and Irwin, 1962).

Age, size, health and nutrition of test animals and their previous exposure to the lethal agent and other environmental factors must be considered before tolerance experiments are performed (Warren, 1971). Our laboratory has used the reference toxicant, dodecyl sodium sulphate (DSS), as one means of periodically checking the health and sensitivity of fingerling rainbow trout, *Salmo gairdneri*. These fish are incorporated into acute lethal toxicity tests with industrial effluents, chemicals and oil dispersants. Dodecyl sodium sulphate was chosen primarily because of its use by LaRoche et al (1970) and their recommendations of its value in standardizing bioassays within labs.

2.2 Chemistry and Toxic Action of DSS

A good reference standard chemical should be sufficiently uncommon in nature that there is little risk that bioassay organisms have been exposed to it or its closely related compounds (Marking, 1966). The chemical or agent should also be a general stressor, resulting in a non-specific integrated biological response. Stressors can be chemicals or physical conditions such as temperature or salinity. According to Alderdice (1963), a primary chemical stressor should have high water solubility, high toxicity, presumed non-specific toxic action, availability as a dry product of high purity and stability (in solution at pH 7.0-8.5) and a facility of quantitative determination.

DSS is a typical anionic detergent (Abel and Skidmore, 1975), with a formula $(C_{12}H_{25})_{12}SO_3Na$. It is commercially available in several forms of purity. It foams when in continuous-flow bioassay (D.G. Alexander, pers. comm.) and in static bioassays in salt water (Wells and Sprague, unpubl. data).

Fish exposed to detergents usually suffer a specific type of gill damage (Abel, 1974; Abel and Skidmore, 1975). They found that DSS shares with phenol and formaldehyde "the capacity to induce extensive cellular death from the earliest stages of poisoning". The toxicity of DSS depends not only on the hardness of water in which the fish are treated but also on the hardness of water in which the fish have previously been acclimatized (Tovell, Newsome and Howes, 1974).

A summary of the acute lethal toxicity of DSS to various organisms (Table 1) shows that 4-day LC50's for six species of fish are in the range 0.5-5.6 mg/l, and for four species of invertebrates are in the range 0.7-108 mg/l. Data are limited and sometimes contradictory. With the one exception where the study showed a relationship between hardness and detergent toxicity, there has been little attempt to determine the effects of abiotic and biotic variables on the toxicity of DSS. Abel (1974) recommends that toxicity tests with detergents include measurement of concentrations in time and space, a complete description of source and purity of the detergent, the toxicity of various degraded states of the detergent and the presentation of toxicity curves allowing a calculation of the incipient lethal levels.

3. LABORATORY TESTS WITH DSS

3.1 Methods and Materials

For all toxicity tests, DSS was laboratory grade (Fisher Scientific Company). It consisted chiefly of sodium lauryl sulphate.

Three types of bioassays were used--static, static-stirred and flow-through. In the case of static and static-stirred bioassays, the concentration of each stock solution was 5 g DSS/ℓ of dilution water. The mixture was stirred gently for 30 min to minimize foaming, then diluted in the test tanks to desired concentrations. All bioassays started within one hour of adding DSS to test tanks.

Static bioassays were performed in rectangular fiberglass tanks lined with 4 mil polyethylene. Volumes of test solution were either 20 ℓ or 60 ℓ, based upon size of fish used for the test. This ensured that oxygen was not depleted, as supplementary aeration was not desired.

TABLE 1 - ACUTE LETHAL TOXICITY OF DSS (DODECYL SODIUM SULPHATE) TO VARIOUS AQUATIC ORGANISMS. ALL CONCENTRATIONS EXPRESSED AS mg/l EXCEPT AS NOTED.

ORGANISM	TOXICITY	REFERENCE
1. Rainbow Trout *Salmo gairdneri*	Died in 40-45 min at 70 ppm (300 ppm hardness). Died after 3 h at 70 ppm (60 ppm hardness).	Tovell et al, 1974
	LT50 in 100 mg/l was 4.9 (3.9-6.1) h to yearling trout.	Abel and Skidmore, 1975
2. Goldfish *Carassius auratus*	Died within 90-110 min at 70 ppm (300 ppm hardness).	Tovell et al, 1974
3. Longnose Killifish *Fundulus similis*	96 h LC50 = 1.8; 0.5	McAuliffe et al, 1975
4. Mummichogs *Fundulus heteroclitus*	96 h LC50 = 4.5-5.6	LaRoche et al, 1970
	96 h LC50 = 1.25	McAuliffe et al, 1975
5. Tidewater Silverside *Menidia beryllina*	96 h LC50 = 1.5	McAuliffe et al, 1975
6. Stickleback *Gasterosteus aculeatus*	96 h LC50 = 0.5; 4.2	McAuliffe et al, 1975
7. Sandworm *Nereis virens*	96 h LC50 = 13.5	LaRoche et al, 1970
8. Brime Shrimp *Artemia salina*	96 h LC50 = 1.5; 5.6	McAuliffe et al, 1975
9. Mysid *Mysidopsis almyra*	24 h LC50 = 2.0	Anderson et al, 1974
10. Grass Shrimp *Palaemonetes pugio*	96 h LC50 = 13.8	McAuliffe et al, 1975
	96 h LC50 = 108	Anderson et al, 1974
11. Lobster Larvae *Homarus americanus*	2 day LC50 = 3.5 (1.8-6.7)	Wells and Sprague, 1975
	4 day LC50 = .7 (.4-1.3)	Wells and Sprague, 1975

The static-stirred bioassays were conducted in rectangular, polyethylene tanks lined with 4 mil polyethylene. The volume of test solution was 160 ℓ. Solutions were stirred by 4-bladed propellors rotated at 430 rpm, and were not aerated.

Stock solutions for the flow-through bioassays were prepared by dissolving 11.42 g DSS in 20ℓ distilled water and stirring overnight in a glass bottle. The solution was dispensed using a modified Mount and Brungs (1967) proportional dilutor. Treatments consisted of 5 duplicated concentrations and 2 controls. The desired concentrations were delivered via glass tubing to rectangular fiberglass test vessels lined with 4 mil polyethylene. Each test vessel contained 20 ℓ solution. Flow rates were 80-120 ml/min and test solutions were not aerated.

Fish stocks were obtained from two hatcheries, Cobequid Trout Hatchery, Collingwood, N.S., and Goosen's Trout Farm, Otterville, Ontario. Mean fish weights, fork lengths, condition coefficients and test solution volume-fish weight ratios per day ranged between 0.7-12.9 gm, 4.0-11.2, 1.0-1.5, and 0.2-10.0, respectively (Appendix Table 1). Initial and final water temperatures, pH and oxygen levels of all test solutions ranged between 13-17 C, 5.9-8.2, and 2.3-11.8 mg/l, respectively (Appendix Table 2).

The diluent was Dartmouth municipal water, dechlorinated by activated carbon and ultraviolet radiation. Typical physico-chemical characteristics of this water are presented in Appendix Table 3.

Bioassay procedures followed Sprague (1973) as closely as possible, and acute toxicities were expressed as LT50's (median lethal times) derived as in Litchfield (1949), and LC50's (median lethal concentrations) derived as in Litchfield and Wilcoxon (1949). Where partial mortalities were limited, LT50's and LC50's are presented as ranges within which mortality occurred. Correlation coefficients on data for LC50 versus fish size were computed using the multiple regression program SPSS (Northwestern University, Chicago).

3.2 Results

Toxicity data from static-stirred bioassays were examined to determine effects of fish batch (Table 2). Sufficient replicate tests to permit examination by batch were available for two of the eight batches tested (74-3 and 75-2). These two groups were significantly different from one another using 95% confidence intervals. Visual examination of these data suggested no trends with time or season. Sufficient data were not available for other bioassay modes to facilitate similar comparisons.

DSS was more toxic to Goosen's rainbow trout in static bioassay mode than in static-stirred and flow-through modes and for Cobequid trout in static mode (Table 3). The toxicity of DSS did not differ for Goosen's flow-through, Goosen's static-stirred and Cobequid static tests. Weight and length varied significantly between Cobequid and Goosen's fish ($P<.001$), the former being much larger fish. Condition coefficients were the same for all groups (Table 3).

Fish weight significantly affected the toxicity of DSS to Cobequid fish ($r=0.67$; $p=0.05$) (Table 4, Fig. 1), as determined using multiple regression analysis. Increased weight was correlated with increased toxicity to Cobequid fish. Toxicity was not correlated with weight, length or condition coefficient of Goosen's fish in any bioassay mode.

Aging of DSS test solutions in open test vessels prior to bioassay reduced their acute toxicity to rainbow trout. LT50's increased from 7 h (95% C.L.=6-8; s=1.13) for fresh test solution to >96 h for test solution aged four days. Toxicity changed markedly between days 3 and 4 (Table 5).

Using a stock of Goosen's trout, an attempt was made to determine whether the toxicity of DSS to the fish changed during an acclimation period of 20 days. Though the data show a possible decrease in toxicity with time, none of the values were significantly different from one another (Table 6).

The variation in acute lethal toxicities of DSS and an oil-plus-oil dispersant mixture was examined (Table 7). The toxicity of DSS varied with the toxicity of the oil-plus-oil dispersant mixture but the changes were in the same proportion. The results of trial 2 (6.5 mg DSS/ℓ) can be taken as a representative value since it is closest to the mean value for the static-stirred mode (6.2 mg/l). The changes in toxic

TABLE 2 - THE TOXICITY OF DSS TO VARIOUS RAINBOW TROUT (*Salmo gairdneri*) BATCHES BIOASSAYED IN THE STATIC-STIRRED MODE BETWEEN JANUARY, 1974, AND MARCH, 1975. ALL TROUT WERE RECEIVED FROM GOOSEN'S TROUT FARM

DATE	BATCH #	LC50	N	$\overline{x}$	95% C.L.
Jan.	74-1	6.5 (range=5.6-7.5)	1	6.5	
Feb.-Mar.	74-2	6.0 (range=4.2-7.5)	1	6.5	
Apr.-May	74-3	6.5 (range=5.6-7.5)			
		8.7 (range=7.5-10.0)			
		8.7 (range=7.5-10.0)			
		7.4 (95% C.L.=6.8-8.1)			
		6.5 (range=5.6-7.5)	5	7.6	1.0
Aug.-Sept.	74-5	4.9 (range=4.2-5.6)	1	4.9	
Oct.-Nov.	74-6	5.6 (95% C.L.=5.4-5.8)			
		6.5 (range=5.6-7.5)	2	6.1	
Dec.-Jan.	74-7	5.8 (95% C.L.=4.5-7.4)			
		4.9 (range=4.2-5.6)	2	5.4	
Jan.	75-1	6.3 (95% C.L.=5.2-7.3)	1	6.3	
Feb.-Mar.	75-2	5.5 (95% C.L.=4.6-6.3)			
		4.5 (range=4.2-4.9)			
		5.3 (range=4.9-5.6)			
		4.9 (range=4.2-5.6)	4	5.1	0.4
	OVERALL		17	6.2	0.6

TABLE 3 - A SUMMARY OF THE ACUTE LETHAL TOXICITY OF DSS TO TWO RAINBOW TROUT (*Salmo gairdneri*) STOCKS TESTED USING DIFFERENT BIOASSAY MODES. 95% CONFIDENCE INTERVALS ARE GIVEN FOR EACH MEAN. NUMBER OF INDEPENDENT BIOASSAYS IS DENOTED BY N

HATCHERY	BIOASSAY MODE	N	LC50 (mg/l)	FISH		
				LENGTH (cm)	WEIGHT (gm)	CONDITION COEFFICIENT
Cobequid	Static	13	5.7±0.4	8.7±0.7	7.9±1.4	1.2±0.0
Goosen's	Static	8	4.3±0.2	5.6±0.8	2.2±1.0	1.2±0.1
Goosen's	Static-Stirred	17	6.2±0.6	5.7±0.5	2.5±0.7	1.1±0.0
Goosen's	Flow-Through	8	5.3±0.5	6.4±0.4	3.6±0.8	1.2±0.1

TABLE 4 - CORRELATION COEFFICIENTS (r) OF LENGTH, WEIGHT AND CONDITION COEFFICIENT WITH THE TOXICITY OF DSS TO TWO STOCKS OF RAINBOW TROUT (*Salmo gairdneri*) TESTED UNDER DIFFERENT BIOASSAY MODES. ANALYSIS CONDUCTED ON UNTRANSFORMED DATA, ASSUMING LINEARITY OF VARIABLES

HATCHERY	BIOASSAY MODE	CORRELATION COEFFICIENTS (r) FOR		
		LENGTH (cm)	WEIGHT (gm)	CONDITION COEFFICIENT
Cobequid	Static	-	0.67*	0.40
Goosen's	Static	0.07	-	0.22
Goosen's	Static-Stirred	0.48	0.37	0.17
Goosen's	Flow-Through	0.69	-	-

* Significant correlation at $p = 0.05$.

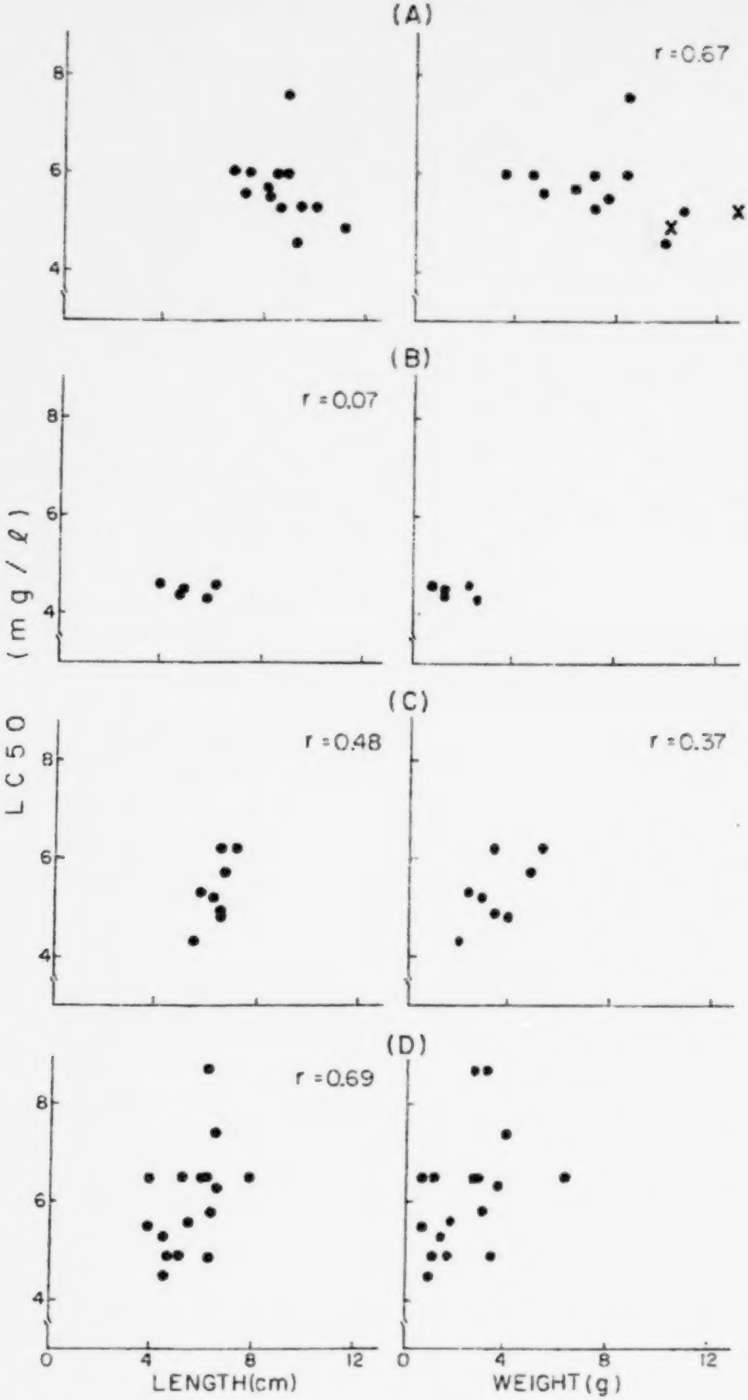

FIGURE 1 - THE EFFECT OF FISH SIZE ON THE TOXICITY OF DSS (DODECYL SODIUM SULPHATE) TO RAINBOW TROUT IN 96-HOUR TESTS AT 15 C. (A) STATIC TESTS USING COBEQUID HATCHERY STOCK, (B) STATIC, (C) FLOW-THROUGH AND (D) STIRRED STATIC TESTS USING GOOSEN'S HATCHERY STOCK

TABLE 5 - THE EFFECT OF AGING ON THE ACUTE LETHAL TOXICITY OF DSS TO RAINBOW TROUT (*Salmo gairdneri*). EACH CONCENTRATION CONTAINED FIVE FISH.

CONC. (mg/l)	AGE OF DSS TEST SOLUTION PRIOR TO BIOASSAY (DAYS)	MORTALITY IN 4 DAYS (%)	LT50 (HR)
7.5	4	0	>96
7.5	3	80	20 (C.L.=17 to 22; s=1.15)*
7.5	2	100	12 (C.L.=10 to 14; s=1.18)
7.5	1	100	12 (C.L.=10 to 14; s=1.18)
7.5	0	100	7 (C.L.=6 to 8; s=1.13)
Control	-	0	>96

* C.L. = 95% confidence interval; s = slope.

TABLE 6 - THE TOXICITY OF DSS DURING A TWENTY DAY ACCLIMATION OF RAINBOW TROUT (*Salmo gairdneri*) FROM HATCHERY TO LABORATORY HOLDING CONDITIONS AND FROM 10 C TO 15 C. DAY 0 IS THE DAY THE FISH WERE RECEIVED AT THE LABORATORY

DAY	LC50 (mg/l)
0	2.5-5.0
5	4.6 (C.L.=4.1 to 5.2; s=1.15)*
11	4.6 (C.L.=4.1 to 5.2; s=1.15)
20	5.3 (C.L.=4.6 to 6.0; s=1.16)

* C.L. = 95% confidence interval; s = slope.

TABLE 7 - COMPARISON OF VARIATIONS OF THE TOXICITY OF DSS AND AN OIL PLUS OIL DISPERSANT MIXTURE. ALL BIOASSAYS WERE IN THE STATIC-STIRRED MODE BUT WITH DIFFERING FISH BATCHES.

HATCHERY	BATCH	TRIAL	LC50 (mg/l) FOR	
			OIL + OIL DISPERSANT	DSS
Goosen's	74-3	1	130 (range=100-180)	8.8 (range=7.5-10.0)
Goosen's	74-1	2	110 (range=56-180)	6.5 (range=5.6-7.5)
Goosen's	75-2	3	88 (range=56-135)	5.5 (range=4.9-6.5)

response to DSS and to the oil-plus-oil dispersant mixture were increased by 26% and 18% respectively (Trial 1) and decreased by 15% and 19% respectively (Trial 3). Different fish stocks were used for different trials but were the same between treatments of the same trial.

In two bioassay tests using Cobequid stock, the test fish were selected from holding tanks where 2% mortality had occurred during the previous 7 day period (data marked "X"; Fig. 1A). A myxobacterial infection, Fin Rot, was also prevalent throughout this stock. These fish did not respond differently from previously tested fish (Fig. 1A).

4. DISCUSSION

The usefulness of DSS as a reference toxicant for rainbow trout depends on its ability to accurately depict their health and their sensitivity to other contaminants under any given situation. Therefore, we must know the purity and stability of the toxicant as well as its effects on test fish in different waters and the influence of variables such as fish size and test population.

In considering the use of DSS as an intra- rather than an inter-laboratory reference toxicant, water chemistry and purity of the compound are reasonably constant as each comes from a single source. The acute toxicity of DSS in solution diminishes with time (Table 3) but since fresh solutions were made up for each bioassay, the rate of degradation between tests is assumed to be similar, and as such, is treated as a constant. Determination of the effects of these variables is vital, however, in considering inter-laboratory calibration tests.

In the experiments presented here, at least two levels of response to DSS are apparent for the three bioassay modes investigated using fish from Goosen's; (a) the static mode (4.5±0.3) and (b) flow-through (5.4±0.5) and static-stirred (6.2±0.6) bioassay modes. Within each group, however, no effect of fish size was observed over the limited size range tested. Fish from Cobequid hatchery on the other hand had a much wider distribution of size and showed increased toxicity to DSS with increased size. Since the fish from Cobequid and

Goosen's hatcheries did not overlap in size range for static bioassays, no comparison of their relative responses to DSS could be made.

The results demonstrate that it is possible to predict that expected values for static-stirred bioassays should fall between 5.6 and 6.8 mg/l DSS. These values are two standard errors above and below the mean value for all tests in that mode. In applying this criterion, we can see that from the data presented in Table 3, we should reject or modify the lowest and two highest results obtained. Similarly, the same criterion could be applied for the static bioassays and flow-through modes. In those cases, fish would be considered as having exhibited a normal response if the LC50 values were within 4.2 to 4.8 and 4.9 to 5.9, for static (Goosen's) and flow-through modes, respectively.

Where toxicity is affected by fish size, as was observed for Cobequid fish, the same criterion can be used. In such cases, the data may be grouped within various fish size ranges. The mean LC50 value and 95% confidence interval for each size range then forms the basis of establishing the normal response curve for the population. However, there is insufficient data presented here to meaningfully perform the exercise.

While changes in the toxicity of DSS to rainbow trout correlate with changes in the sensitivity of the fish to an oil-plus-oil dispersant mixture, do changes in their sensitivity to DSS also provide insight into the health of fish stocks? In these experiments, we tested fish that had just arrived at our laboratory and again after 5, 11 and 20 days. These fish were presumed to be stressed following several hours shipment in a small carton and having to adjust to a temperature change from 10 C to 15 C. Yet, there was no obvious difference between the initial and final response to DSS. Similarly, a fish stock, infected with Fin Rot while in holding tanks, where 2% mortality had occurred within a 7 day period, showed the same response to DSS as did other fish in the same size range (see Fig. 1A for weight, noting the values marked "X").

There are several possibilities that could explain the observed phenomenon. Firstly, it is possible that the fish were in stress but at a level below what could be detected using DSS. Secondly, transportation and small temperature shifts did not stress the fish. And thirdly, the adjustments made by fish to changes in habitat and temperature did not affect the same biological systems that were affected by DSS.

DSS acts on the gills of fish (Abel and Skidmore, 1975). If its action is not modified by metabolic activity (e.g. if the damaging action is dependent on surface contact but not on transport across membranes), then changes in sensitivity related to the metabolic activity of fish will not be detected using DSS.

Though the actual level of stress to the fish was not measured, it is generally accepted that fish are stressed during transportation. This stress is generally reflected in higher metabolic rates in fish. A trend towards reduced toxicity was in fact observed with time (Table 6), though the differences in values between the start and end of the experiment were not significant. The 5 C temperature increase from 10 C to 15 C experienced by the fish just after being transported would have, if anything, added to the increase in metabolic rate. This suggests that DSS may not be sensitive enough to detect general stresses of that magnitude or of that type.

The significance to the health of fish of 2% mortality in 7 days in the holding stocks is unknown. Nor is it known to what extent the observed level of infection by Fin Rot affected fish health. The usefulness of DSS toxicity as a general health indicator for rainbow trout cannot be established. The authors however consider that mortality and infections of fish are indicative of decline or pending decline in the health of the stock as a whole.

How, then, does DSS relate to the four functions noted earlier for a good reference toxicant? DSS seems capable of measuring changes in sensitivity (Function 1) to oil-plus-oil dispersant mixtures. It is likely that DSS, a surfactant, has the same mode of toxic action as oil dispersants which contain surfactants. Hence DSS is useful as a reference toxicant for toxicants having a similar mode of toxic action.

Based on our work, DSS may not be a good indicator of factors affecting general fish health and sensitivity (Function 1). This was shown by a lack of response to DSS by fish infected with Fin Rot or stressed by transportation and changes in temperature. Because of this, its use to establish and define standard testing conditions (Function 2), to act as an internal standard for comparing relative toxicities (Function 3) for toxicants having a different mode of toxic action, or to act as an interlaboratory reference toxicant (Function 4), would be similarly limited. Surprisingly, while DSS has often been advocated or required as a reference toxicant (LaRoche et al, 1970; Anon, 1973; LaRoche, 1974; Gentile and Johnson, 1974), verification of its usefulness and application has not been documented.

It is unrealistic to expect that a single response to a chemical can demonstrate the sensitivity and health of fish. It might prove advantageous to develop and use a series of tests (using biochemical, physiological and behavioural measurements) that depict the normality of various vital systems within a test organism. Similar approaches have been suggested before (Waldichuk, 1970; Sprague, 1971; and many others) but as yet, no quantitative diagnostic system has been developed for fish.

The requirement for simultaneous testing of reference chemicals and an unknown toxicant is often unacceptable. Volumes of the unknown may be limited, thereby precluding more than one test and possibly resulting in lost information if the test fish are proven unacceptable. Furthermore, reference tests should be performed within a day. Given an acceptability response, the test on the unknown could follow immediately with reasonable assurance of a normal response. Pretests of short duration will also result in considerable savings of resources.

The value of DSS as an intralaboratory toxicant is probably limited to identifying changes in sensitivity of fish to toxicants having a toxic action similar to DSS. Meaningful reference testing systems should depict the normality of various vital systems to establish health and sensitivity of test fish. Reference test systems should also be capable of rapidly providing the necessary information prior to testing unknown toxicants.

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APPENDIX TABLE 1 - MEANS (±S.E.) OF FISH WEIGHTS, LENGTHS AND CONDITION COEFFICIENTS AND RATIOS OF LITRES OF TESTWATER PER GRAM OF FISH PER DAY FOR CONTROL AND TOXICITY TESTS ON D.S.S.

BATCH	DATE TEST STARTED	MEAN FISH WEIGHT (±S.E.), gm.	MEAN FISH LENGTH (±S.E.), cm.	MEAN CONDITION COEFFICIENT (±S.E.), $wt/\ell^3 \times 100$	ℓ/gm/day	BIOASSAY MODE
73-1	10/09/73	3.7±0.3	6.8±0.2	1.1±0.0	0.3	Static
73-1	17/09/73	5.1±0.3	7.2±0.1	1.3±0.0	0.2	Static
73-1	26/09/73	4.7±0.3	7.4±0.2	1.2±0.0	0.2	Static
73-1	02/10/73	7.7±0.9	8.2±0.3	1.3±0.0	0.4	Static
73-1	15/10/73	8.5±0.6	8.9±0.2	1.2±0.0	0.4	Static
73-1	22/10/73	6.4±0.4	8.1±0.2	1.2±0.0	0.5	Static
73-1	26/10/73	7.9±0.4	8.7±0.1	1.2±0.0	1.6	Cont. Flow
73-1	06/11/73	10.0±0.6	9.3±0.2	1.2±0.0	0.3	Static
73-1	07/11/73	9.9±0.9	9.3±0.3	1.2±0.0	1.3	Cont. Flow
73-1	15/11/73	10.6±0.5	9.9±0.2	1.1±0.0	0.4	Stirred Static
73-2	23/11/73	7.1±0.7	8.5±0.3	1.1±0.0	0.7	Static
73-2	28/11/73	8.4±0.5	9.2±0.2	1.0±0.1	0.5	Stirred Static
73-2	03/12/73	10.0±0.5	9.5±0.2	1.1±0.0	0.4	Stirred Static
73-2	03/12/73	10.2±0.5	11.2±1.9	1.2±0.0	0.3	Static
73-2	03/12/73	12.9±1.5	10.1±0.3	1.2±0.0	0.2	Static
73-3	21/11/73	7.1±0.4	8.5±0.2	1.0±0.0	0.6	Stirred Static

Cont'd

APPENDIX TABLE 1 - CONT'D

BATCH	DATE TEST STARTED	MEAN FISH WEIGHT (±S.E.), gm.	MEAN FISH LENGTH (±S.E.), cm.	MEAN CONDITION COEFFICIENT (±S.E.), wt/ℓ^3 x 100	ℓ/gm/day	BIOASSAY MODE
73-5	10/12/73	6.5±0.4	8.5±0.2	1.1±0.0	1.2	Stirred Static
73-5	10/12/73	8.5±0.7	8.9±0.2	1.1±0.0	0.4	Static
73-5	10/12/73	7.1±0.5	8.6±0.2	1.1±0.0	0.4	Static
73-5	10/01/74	10.7±1.2	4.4±0.3	1.2±0.0	0.3	Static
74-1	05/02/74	0.8±0.1	4.1±0.1	1.1±0.1	1.3	Static
74-1	05/02/74	0.7±0.1	4.0±0.1	1.1±0.1	5.7	Stirred Static
74-2	05/03/74	1.2±0.1	5.3±0.5	1.0±0.0	3.3	Stirred Static
74-2	05/03/74	1.4±0.1	5.0±0.1	1.1±0.0	0.7	Static
74-2	02/04/74	2.6±0.3	5.9±0.2	1.2±0.0	0.4	Static
74-3	23/04/74	3.0±0.2	6.1±0.1	1.3±0.0	2.7	Stirred Static
74-3	02/05/74	3.5±0.2	6.6±0.1	1.2±0.0	3.8	Cont. Flow
74-3	07/05/74	3.5±0.2	6.6±0.1	1.1±0.0	3.7	Cont. Flow
74-3	09/05/74	2.8±0.2	6.3±0.2	1.0±0.1	2.9	Stirred Static
74-3	13/05/74	3.0±0.2	6.3±0.1	1.2±0.0	4.3	Cont. Flow
74-3	16/05/74	3.3±0.3	6.5±0.2	1.2±0.0	2.4	Stirred Static
74-3	22/05/74	4.1±0.3	6.6±0.3	1.2±0.0	2.0	Stirred Static
74-3	22/05/74	5.4±0.4	7.2±0.1	1.1±0.0	2.4	Cont. Flow
74-3	28/05/74	6.4±0.4	7.9±0.2	1.2±0.0	1.2	Stirred Static

Cont'd

ATTENDIX TABLE 1 - CONT'D

BATCH	DATE TEST STARTED	MEAN FISH WEIGHT (±S.E.), gm.	MEAN FISH LENGTH (±S.E.), cm.	MEAN CONDITION COEFFICIENT (±S.E.), wt/ℓ^3 x 100	ℓ/gm/day	BIOASSAY MODE
74-4	02/05/74	1.5±0.1	5.0±0.1	1.2±0.0	8.8	Cont. Flow
74-4	07/05/74	1.3±0.1	5.0±0.1	1.1±0.0	10.0	Cont. Flow
74-4	13/05/74	1.6±0.1	5.2±0.1	1.1±0.0	8.1	Cont. Flow
74-4	22/05/74	2.4±0.1	5.8±0.1	1.2±0.0	5.3	Cont. Flow
74-4	18/06/74	4.1±0.2	6.6±0.1	1.3±0.0	3.2	Cont. Flow
74-4	24/06/74	4.9±0.2	6.7±0.1	1.5±0.0	2.7	Cont. Flow
74-5	07/08/74	1.2±0.1	4.9±0.5	1.3±0.0	2.6	Static
74-5	17/08/74	1.2±0.1	4.7±0.1	1.2±0.1	3.3	Stirred Static
74-5	04/09/74	2.1±0.1	5.6±0.1	1.2±0.0	6.3	Cont. Flow
74-6	12/10/74	1.9±0.2	5.5±0.1	1.1±0.1	4.2	Stirred Static
74-6	30/10/74	2.3±0.2	6.2±0.1	1.2±0.1	0.4	Static
74-6	07/11/74	2.9±0.2	6.2±0.1	1.2±0.1	2.7	Stirred Static
74-7	30/10/74	1.3±0.1	4.8±0.1	1.1±0.1	0.8	Static
74-7	02/01/75	3.2±0.2	6.4±0.6	1.1±0.1	2.5	Stirred Static
74-7	08/01/75	3.6±0.9	6.3±0.4	1.2±0.0	2.2	Stirred Static
75-1	29/01/75	3.8±0.3	6.6±0.4	1.0±0.1	2.1	Stirred Static
75-1	06/02/75	5.0±0.4	7.6±0.2	1.1±0.0	0.3	Static

Cont'd

APPENDIX TABLE 1 - CONT'D

BATCH	DATE TEST STARTED	MEAN FISH WEIGHT (±S.E.), gm.	MEAN FISH LENGTH (±S.E.), cm.	MEAN CONDITION COEFFICIENT (±S.E.), wt/ℓ^3 x 100	ℓ/gm/day	BIOASSAY MODE
75-2	24/02/75	0.7±0.0	4.0±0.1	1.0±0.0	5.7	Stirred Static
75-2	03/03/75	1.0±0.1	4.6±0.1	1.0±0.0	3.9	Stirred Static
75-2	17/03/75	1.5±0.1	4.6±0.2	1.0±0.0	2.7	Stirred Static
75-2	24/03/75	1.8±0.1	5.1±0.7	1.1±0.0	2.8	Stirred Static
75-6	05/08/75	3.2±0.3	6.3±0.2	1.2±0.0	0.6	Static

APPENDIX TABLE 2 - RANGES OF PHYSICO-CHEMICAL PARAMETERS FOR TEST SOLUTIONS OF D.S.S.

DATE TEST STARTED	TEMP., °C	pH	D.O., mg/1	BIOASSAY MODE
10/09/73	14-15	6.4-7.1	5.1-8.2	Static
17/09/73	14-15	6.2-7.5	2.9-9.5	Static
26/09/73	14-15	6.5-7.3	3.8-8.6	Static
02/10/73	15-16	6.2-7.5	5.3-9.3	Static
15/10/73	14.5-15	6.7-7.9	2.6-10.0	Static
22/10/73	14-15	6.6-7.1	5.7-10.1	Static
26/10/73	14-15	7.1-7.6	7.7-10.0	Cont. Flow
06/11/73	14-16	6.5-6.8	4.5-10.0	Static
07/11/73	14-16	6.5-7.5	8.2-9.9	Cont. Flow
15/11/73	12-16	6.9-7.6	9.2-10.4	Stirred Static
23/11/73	14.5-16	6.9-7.5	2.3-11.8	Static
28/11/73	14-16	6.5-7.0	10.1-11.0	Stirred Static
03/12/73	14-15	6.5-6.9	9.0-11.2	Stirred Static
03/12/73	14.5-16	6.5-7.0	3.5-10.4	Static
03/12/73	14-16	6.1-6.8	3.4-11.0	Static
21/11/73	14-16	7.0-7.4	9.2-11.4	Stirred Static
10/12/73	14-15	6.7-7.0	9.0-10.1	Stirred Static
10/12/73	14-15	6.4-6.9	6.5-10.1	Static
10/12/73	14-15	6.4-7.0	3.9-10.3	Static
03/01/74	14-15	6.4-6.9	5.8-10.3	Static
05/02/74	14-15	6.9-7.3	7.1-10.2	Static
05/02/74	14-15	7.0-7.5	8.8-10.2	Stirred Static
05/03/74	14.5-16	6.8-7.1	10.1-10.4	Stirred Static
05/03/74	14.5-16	6.5-7.0	8.1-10.8	Static
02/04/74	15-15	6.6	9.0-10.2	Static
23/04/74	14-16	6.5-6.9	9.4-9.8	Stirred Static
02/05/74	15-16	6.4-6.9	7.9-10.8	Cont. Flow
07/05/74	15-16	6.6-6.9	9.2-10.6	Cont. Flow
09/05/74	15-16	6.5-7.0	8.8-11.2	Stirred Static
13/05/74	15-17	6.5-6.7	8.6-10.1	Cont. Flow
16/05/74	15-16	6.7-7.1	8.2-9.7	Stirred Static
22/05/74	15-16	6.3-6.8	5.7-10.1	Stirred Static
22/05/74	15-17	6.8-7.5	9.0-9.7	Cont. Flow
28/05/74	15-16.5	6.8-7.0	7.9-10.1	Stirred Static

Cont'd

APPENDIX TABLE 2 - CONT'D

DATE TEST STARTED	TEMP., °C	pH	D.O., mg/l	BIOASSAY MODE
02/05/74	15-16	6.5-6.9	9.1-11.1	Cont. Flow
07/05/74	15-16	6.6-6.8	9.8-10.2	Cont. Flow
13/05/74	15-17	6.4-6.9	9.2-10.4	Cont. Flow
22/05/74	14-15	6.3-6.9	8.9-9.7	Cont. Flow
18/06/74	14-15	6.8-8.2	8.4-9.5	Cont. Flow
24/06/74	14.5-16	6.6-7.2	6.4-9.9	Cont. Flow
07/08/74	15-16	6.6-7.2	7.9-9.5	Static
17/08/74	15-16	6.6-6.8	8.9-9.8	Stirred Static
04/09/74	15-16	6.3-6.8	7.9-9.2	Cont. Flow
12/10/74	15-16	6.2-7.0	9.1-10.4	Stirred Static
30/10/74	14.5-16	6.1-6.5	6.9-8.5	Static
07/11/74	15-16	6.5-6.9	9.1-9.9	Stirred Static
30/10/74	15-15	6.2-6.6	7.3-8.6	Static
02/01/75	15-15	5.9-6.3	8.8-9.3	Stirred Static
08/01/75	15-16	5.9-6.2	9.3-9.8	Stirred Static
29/01/75	15-17	6.3-6.5	9.4-9.8	Stirred Static
06/02/75	15-16	6.1-7.0	5.5-10.2	Static
24/02/75	14-16	6.3-6.8	9.6-10.4	Stirred Static
03/03/75	14-16	6.4-6.6	9.4-10.6	Stirred Static
17/03/75	14-15	6.2-7.2	9.2-10.2	Stirred Static
24/03/75	14-16	6.3-6.6	8.4-10.0	Stirred Static
05/08/75	14-16	6.0-7.1	5.0-11.0	Static

APPENDIX TABLE 3 - CHARACTERISTICS OF DECHLORINATED DARTMOUTH MUNICIPAL FRESH WATER AT BEDFORD INSTITUTE OF OCEANOGRAPHY, JANUARY - FEBRUARY, 1975

WATER QUALITY PARAMETERS

PARAMETER	UNITS	JANUARY			FEBRUARY		
		$\bar{x}$ [1]	σ [2]	RANGE	$\bar{x}$	σ	RANGE
Acidity (titrimetric method [Ammanval])	mg/l $CaCO_3$	5.37 (4)	0.84	4.49-6.40	4.39 (2)	0.14	4.29-4.49
Alkalinity ($CaCO_3$)	ppm	- (4)	-	<1-3.1	4.0 (3)	0.06	3.9-4
Chlorine	ppb	<10 (3)	0	<10			
Copper	mg/l	- (3)	-	<.001-.003	- (3)	-	<.001-.005
Hardness, Scale From (CaX2.5) + (MgX4.1)	mg/l	13.42 (4)	1.21	12.13-14.72	14.99 (2)	0.31	14.77-15.21
Humic Acids	ppm	- (3)	-	<1-2.6	- (3)	-	<1-2.7
Inorganic Carbon	ppm	<1 (4)	0	<1	<1 (3)	0	<1
Iron	mg/l	0.11 (4)	0.08	0.003-0.18	0.119 (3)	0.016	.101-0.13
Lead	mg/l	- (3)	-	<.001-.002	- (3)	-	<.001-0.02
Nitrogen: Nitrite	ppm	<.005 (4)	0	<.005	<.005 (3)	0	<.005
Nitrate	ppm	0.028 (4)	0.016	.005-.038	0.057 (3)	0.007	0.05-0.064
Nitrite & Nitrate	ppm	- -	-	-	-	-	-
Oxygen (Tank 1)	mg/l	9.48 (4)	0.60	8.7-10.0	8.95 (2)	1.34	8.0-9.9
pH		6.32 (4)	0.22	6.12-6.6	6.55 (2)	0	6.54-6.55
Phosphate (total)	ppm	<.005 (4)	0	<.005	<.005 (3)	0	<.005
Total Carbon	ppm	- (4)	-	<1-2.5	<1 (3)	0	<1
Total Dissolved Solids	mg/l	41 (3)	10.2	30-50	60.7 (3)	7.4	55-69
Turbidity	jtv	0.50 (4)	0.14	.4-.7	0.67 (3)	0.46	0.32-1.2
Zinc	mg/l	-	-	<.001-.003	- (3)	-	<.001-.004

[1] $\bar{x}$ - Mean.

[2] σ - Standard deviation of mean.

DISCUSSION OF REFERENCE TOXICANTS

Ray Brouzes

I think this is a very useful and timely topic, but there is a shortcoming in all of this. The testing does not indicate whether weakened or moribund animals can be identified. All tests have dealt with healthy animals and a reproducible response has been illustrated. Until weakened individuals can be identified, I do not think these chemicals are very useful.

Ed Pessah

I agree. D.S.S. is not a very useful reference chemical. The mode of toxic action is critical. I did mention that we are only capable of picking up things that act on the gills which is the site of action for D.S.S. I am suggesting that there is some mechanical and chemical action at the gill surface rather than metabolic action. The other thing is that the test should be a decisive test.

Ray Brouzes

Perhaps one or more chemicals will be required that can give a rapid medical check on the specimens.

A whole arsenal of chemicals may be required to indicate whether the test organisms have defective liver, kidney, heart or respiratory systems. Routine research is not bad, but if you are bringing me to court, I am certainly going to defend myself by challenging your results and I think you would want to have them as foolproof as possible.

Jack Klaverkamp

We need a number of toxicologists thinking about this question. I would agree with Ed to avoid the use of something like D.S.S. John Davis's recent article concludes that the usefulness of reference toxicants may be confined to detecting large differences and sensitivities of test fish, which I strongly support. One of the things I like intuitively about phenol and sodium azide is that, if one of these large differences that John talks about such as a metabolic dysfunction is present, these chemicals are non-specific and act on just about all organ systems. There was a paper published in 1967 by some Polish workers who described the physical pathology of phenols wiping out everything. Sodium azide also affects all cells that respire. Therefore, theoretically they should pick up liver and kidney dysfunction. The challenge is, to induce some of these large biological differences and then test the reference chemicals.

We hope to artificially induce disease and stresses, and maybe next year we might be here talking about those experiments.

Ray Brouzes

When you use a systemic poison such as phenol I doubt that you are able to detect a healthy versus a non-healthy organism. The effect is so massive and non-specific that you won't distinguish the difference.

Jack Klaverkamp

The thing about phenol is that it has a very sharp point between lethality and non-lethality. Theoretically, because of that sharp cut-off point, that massiveness that you describe really won't apply.

Gary Alexander

We have completed tests on manipulated fish stocks. We chose three stresses incorporated in transporting fish by plane. The stresses included low oxygen, temperature variation, buffetting and crowding. We observed 17% mortality in the crowded stress condition, versus 2% under control conditions. Strain differences were also examined. Two strains of rainbow trout from Squally, Idaho were simultaneously exposed to copper sulphate, sodium azide, phenol, sodium pentachlorophenate and D.S.S. and median survival times were compared. After 72 tests we have observed that phenol consistently distinguishes between stressed and non-stressed fish while D.S.S. indicated no differences among the treatment groups.

Don McLeay

Reference toxicants can serve two uses. One use is to carry out comparisons within a single laboratory of different fish stocks to be used for experimental purposes. In this regard I feel that the choice of a reference toxicant is not too critical as long as it is highly soluble, quite toxic and stable during the test. We have used a number of different chemicals including heavy metals. As long as the water quality conditions are stable for the duration of the test, heavy metals are as good as anything and the concentrations can be confirmed by AAS. Secondly, they may be used to compare test results with other laboratories. It is essential that screening procedures for a reference toxicant include testing in different dilution waters which vary in water quality characteristics such as pH, hardness and conductivity. If a wide range of test results are produced using one stock of fish at one particular time then obviously that reference toxicant is not useful for inter-laboratory comparisons.

There are other ways of measuring the condition of fish such as recording the residual oxygen at the time of death or the temperature tolerance of the fish stock. I would think that these parameters are less susceptible to variations in water quality. Residual oxygen tests have been investigated by a number of people including Gary Vickers, E.V.S. Consultants, John Davis and myself. It seems to be a very repeatable test and requires only 4-8 hours to complete.

Jack Klaverkamp

I, in no way support the use of chemicals to the exclusion of the physical characteristics of the D.O. and temperature. I would disagree with you, that it is not important as to what chemical we select. Gary Alexander stated that some artificially induced stresses were identified with phenol but not with D.S.S. I would like to see the addition of a metal because azide provides an anion, phenol is non-ionic and the metal would provide cation. Brown (1967) showed that phenol is not influenced by water hardness, whereas anionic or cationic substances are.

Peter Hodson

I find it strange that we are talking about a medical check-up where the patient dies. Perhaps we should be looking at the physiological characteristics of normal fish. The physiological condition of laboratory stocks could then be compared to the normal values.

Ed Pessah

The reference test is not the end product. I think that if we spend an awful lot of time on the reference test then it only takes away from time devoted to the main program. Quick tests are necessary on live animals, they need to be quick and they need to be carried out before the definitive work.

John Davis

I listened with great interest to all these things because they are really the concepts we have been wrestling with for a couple of years now. I myself see two potential uses for reference toxicants, firstly, the comparison of results among laboratories across the country and secondly, the classification of reference toxicants as to site of action and the exposure of fish stocks according to type of chemical that will ultimately be tested. If a specific toxicant affects gas exchange across the gills it would be appropriate to have reference toxicants that have that same affect. We should try to not only look at the toxicity but also the mode of toxicity.

Jack Klaverkamp

Knowledge of the mode of action of many chemicals is sparse to say the least. Hopefully, five years from now we would have that type of information but not today.

Ed Pessah

The other thing that we have to look at is the criteria for accepting, rejecting of modifying data for the unknowns. I find it difficult to accept the use of site specific reference toxicants only, since there may be other parameters that will affect mortality. Therefore, while the test stocks may be normal with reference to one physiological function, they may not be normal with reference to the overall population of healthy fish stocks.

Gary Vigers

I would like to suggest a different approach for the selection of reference toxicants. The objective here is to ultimately develop a test for industrial applications and regulations, implementation or compliance. Would not an empirical basis of selecting industry specific reference toxicants be a more suitable approach? We know the characteristics of the contaminants in the pulp and paper mill effluents. Why would it be necessary to define the mode of action of these toxicants prior to implementing them for reference use?

Jack Klaverkamp

John Davis said that one of the uses of reference toxicants was for inter-laboratory comparisons. He said that it was rather naive and I think that the previous suggestion is too. It might serve better as an internal check. I would also disagree when you say reference toxicants should be restricted to industrial applications. I would like to see the day when you read any published report on the toxicity of some new insecticide for instance, that somewhere in the methods the LC-50 of a reference toxicant is reported just as the D.O., pH and temperature is reported. It would also have to be reported that the reference toxicant value is representative for the fish stocks used in that laboratory.

Gary Vickers

I don't think a universal reference toxicant will ever be implemented. What I am suggesting is that we should have a supplementary list of chemicals to cover specific industries so we would possibly be looking at two reference toxicants. Industry would realize that the fish stocks have been tested with an industry specific compound and know that the response is valid for that particular industry. We don't know that with D.S.S. or some other chemicals.

Ed Pessah

I don't think we have to go that way. We have to decide what constitutes a healthy fish then run the definitive test. I don't know that we are going to do it with one or two chemicals. The name of the game is to identify before you do your test whether that fish is healthy.

Cecil Inniss

Since I was intending to chair the session on Stephan's paper this afternoon I was wondering how we would deal with something so encyclopedic. Perhaps we should narrow the discussion to reference toxicants.

Whether it is possible to compare laboratories or not, I think it is possible to compare the mediums from laboratories. If certain laboratories consistently have poor quality fish, I think that this will show up in a reference toxicant test.

I am proposing this afternoon, to narrow the topic to reference toxicants with a view to coming to some sort of resolution. People here from various laboratories will then return to their laboratories, hopefully, and look at not reference toxicants in general, but at a selected number of chemicals. I would also propose that we exclude chemicals like dehydroabiatic acid and D.S.S. for the simple reason that it is almost impossible in many of these cases to define what you are testing.

We had a routine situation here in which we tested sodium pentachlorophenate as used in a fungicide and we found that we could make no accurate dilutions since the fish were less sensitive to sodium pentachlorophenate than the analytical instruments. The gas chromatography group had to dilute our dilutions. What were we testing, the accuracy of dilutions or solutions?

I had chosen CsCl which unfortunately had two disadvantages. It did not kill fish very well and it was very expensive.

Reference

Brown, V.M., D.G. Shuben and J.K. Fawell. 1967. "The acute toxicity of phenol to rainbow trout in saline waters;" Water Research 1:683-685.

TOPIC III

SUBLETHAL BIOASSAY

Short-term indicators of long-term sublethal effects of metals on fish.[1]

by

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[1] Presented at the 2nd Annual Toxicity Workshop, Ontario Ministry of the Environment, Rexdale, Ontario. November 4, 1975.

Water quality criteria for aquatic organisms define the maximum or minimum environmental requirements for the maintenance of healthy populations. For toxicants, criteria based solely on lethal concentrations would not adequately protect an organism if sublethal concentrations inhibited reproduction. In this case, the population could disappear without evident direct mortality. Therefore the criterion for a specific toxicant must be the maximum concentration permitting successful completion of life cycles. I shall call this the safe concentration. Any concentration preventing successful completion is unsafe. The studies of fathead minnow (Pimephales promelas) life cycles, pioneered by D. I. Mount, U.S. EPA, have provided the basis for a large number of metal criteria.

Life cycle studies, if carried over several generations, may take years to complete and the information may apply to only one organism in one water type. Evaluation of the interactions of mixtures, of the effect of changing the chemical characteristics of water, or of the response of different species, requires either repetition of the experiment or use of some short-cut method that estimates safe concentrations. The physiological responses of fish to toxicants provide some possible short-cut methods. The requirements for these methods are as follows:

1) a completed life cycle experiment must be available for comparison of results. As Mount and Stephen (1967) stated, even a good physiologist cannot envisage the impact of a 10% change in hematocrit.
2) Metal specificity - other experimental variables or toxicants should not produce the same response.

3) consistent response - the response should be repeatable and measurable under a variety of situations.

4) the response should occur within 6 months to provide savings in resources.

5) The response should occur and be easily measured in other species.

6) The response should occur at concentrations close to the safe concentration.

Numerous short-cut methods to evaluate sublethal metal toxicity are reported in the literature. The simplest and cheapest are 96-hour LC50's with application factors. However, there are several drawbacks. The first is the multiplication of errors in the estimate of safe concentrations. Secondly, 96-hour LC50's may vary dramatically with the chemical characteristics of water since LC50's are often near solubility limits. The safe concentration, however, may be relatively constant. Consequently, an application factor derived for one water type is not correct for another.

Behaviour of fish (Table 1)

A second method evaluates behavioural responses of exposed fish. Supposedly an organism's behaviour "represents the final integrated result of a diversity of biochemical and physiological processes" (Warner et al, 1966). Therefore, any deviation from normal behaviour is an expression of an adverse physiological reaction.

Movement patterns, learning, avoidance, locomotor, and feeding behaviour have been quantitatively compared to metal exposures. Kleerekoper et al (1972, '73) have shown that as little as 10-17 μg/l copper can quickly change movement patterns of goldfish (*Carassius auratus*). However, the cost and complexity of the approach prohibit general application.

Table 1

Metals that affect behaviour of fish

Metal	Parameter	Fish	Minimum Effective Concentration ($\mu g/l$)	Exposure Time	Safe-unsafe Concentration Range ($\mu g/l$)	96 hour LC50 ($\mu g/l$)	Author
Copper	Movement patterns	Goldfish	10-17	< 24 h	?	?	Kleerekoper *et al* 1972, 1973
Arsenic	Learning behavior	Goldfish	100	48 h	?	32,000 (7-day)	Weir & Hine, 1970
Mercury			3	"	?	820	
Lead			70	"	?	110,000	
Selenium			250	"	?	12,000	
Copper	Avoidance - lab	Atlantic salmon	2.4	< 24 h	?	43	Sprague *et al*, 1965
Zinc			54	< 24 h	?	600	
Copper	Avoidance - field	Atlantic salmon	≈ 19	?	?	48	Sprague *et al*, 1965.
Zinc			≈ 240	?	?	600	
Copper	Locomotor Activity	Brook trout	6	< 1 h	9.5 - 17.4	110	Drummond *et al*, 1973
Copper	Feeding Activity	Brook trout	9	< 24 h	9.5 - 17.4	110	Drummond *et al*, 1973

Goldfish learning behaviour was significantly impaired by arsenic, mercury, lead and selenium at 100, 3, 70 and 250 µg/l respectively (Weir and Hine, 1970). Learning was evaluated by use of conditioned stimuli and the experimental design appears straightforward. The basic drawback of these studies is that there are no reported safe concentrations of the metals for goldfish. Consequently, there is no way of judging whether these methods give an accurate estimate of the safe concentration.

Upstream migration of Atlantic salmon (Salmo salar) was reversed at copper and zinc concentrations above 19 and 240 µg/l respectively (Sprague et al, 1965). It would appear that these lab results require an application factor for prediction of safe concentrations.

Drummond et al, (1973) demonstrated that locomotor activity of brook trout (Salvelinus fontinalis) increased within minutes of exposure to concentrations of copper as low as 6 µg/l. In addition, feeding activity decreased for up to 24 hours at concentrations as low as 9 µg/l. These fast responding behavioural parameters predict a safe concentration for copper of about 6-9 µg/l. Since the safe concentration based on reproduction was 9.5 µg/l, these parameters satisfy some of the requirements for a short-term indicator of long-term effects.

Metabolism (Table 2)

Cadmium and zinc, in vitro, reduced oxygen metabolism of bluegill (Lepomis macrochirus) liver mitochondria at concentrations of 3.3×10^{-3} and 3.3×10^{-2} µMoles/ml reaction mixture respectively (Hiltibran, 1971).

Table 2

Metals that affect metabolism of fish.

Metal	Parameter	Fish	Minimum Effective Concentration ($\mu g/l$)	Exposure Time	Safe-unsafe Concentration Range ($\mu g/l$)	96 hour LC50 ($\mu g/l$)	Author
Cadmium Zinc	Respiration of Bluegill liver mitochondria	Bluegills	3710 *in vitro* 2145 *in vitro*	several hours	?	1940 ** 4200 **	Hiltibran, 1971
Copper	Warburg O_2 consumption of hepatopancreas	Crayfish	635.7 *in vitro* 5000 *in vivo*	2 1/2 h 48-72 h	?	3000	Hubschman, 1967
Cadmium Copper	Oxidation of lactate by gills	Rainbow trout	1120 64	24 h 48 h	? ?	10 (7-day)** 6.4 - 64	Bilinski and Jonas, 1973
Methyl mercuric chloride	O_2 consumption by gills	Rainbow trout	10	12 wks	1-3*	?	O'Connor and Fromm, 1975

* Drummond *et al*, 1974 for brook trout

** NAS, 1974

While these data are interesting, they cannot estimate safe concentrations without a comparison of cellular metal concentrations to metal exposure.

Hubschman (1967) measured Warburg oxygen consumption of crayfish (*Orconectes rusticus*) hepatopancreas exposed to copper *in vitro* and *in vivo*. Endogenous respiration was inhibited by 10^{-2} M copper in the reaction mixture while succinate respiration was inhibited by 10^{-5} M. Endogenous respiration of hepatopancreas tissue from crayfish exposed *in vivo* was unaffected by 5 mg/l copper whereas succinate respiration was inhibited after 48 to 72 hours exposure. This concentration of copper was lethal for crayfish and there was no relationship of tissue residues of copper to exposure concentration.

Oxidation of lactate by rainbow trout (*Salmo gairdneri*) gills was inhibited by exposure of the fish *in vivo* to 1.12 mg/l cadmium for 24 hours or .064 mg/l copper for 48 hours (Bilinski and Jonas, 1973). However, lower metal concentrations, while still lethal, did not affect oxidative metabolism despite longer exposure times. Therefore this method does not appear sensitive enough for estimation of safe concentrations.

O'Connor and Fromm (1975) measured gill oxygen consumption of rainbow trout after exposure to 10 μg/l of methyl mercuric chloride for up to 12 weeks. The safe concentration for trout is about 1 μg/l (Drummond *et al*. 1974) so that this approach requires a small application factor.

Breathing pattern (Table 3)

Breathing rates of bluegills, as measured by movement of opercula, was changed by 2.55 mg/l zinc (Sparks et al, 1972). The safe concentration of zinc for bluegills was between .076 mg/l (no effect) and .235 mg/l (reproduction inhibited) (Sparks et al, 1972). A drawback to this method is that bluegill breathing rates are easily disturbed so that fairly expensive facilities are required for isolation from noise and vibration (Sparks et al, 1972).

Morgan and Kühn (1974) measured the effects of copper and cadmium on breathing rates of largemouth bass (*Micropterus salmoides*). Although concentrations as low as 0.5 mg/l cadmium and 0.1 mg/l copper were effective, the response was not large and the concentrations are close to lethal.

Drummond et al (1973) observed significant increases in cough frequency of brook trout at 9 μg/l copper, a concentration close to the safe level of 9.5 μg/l. Therefore, breathing rates per se do not provide a sensitive estimate of safe concentrations of metals. However, components of the breathing pattern, such as coughing, appear to directly estimate safe concentrations.

Blood chemistry (Table 4)

O'Connor and Fromm (1975) measured a significant increase in hematocrit of rainbow trout exposed for 12 weeks to 10 μg/l of methyl mercuric chloride. However, they saw no change in plasma sodium, potassium, chloride, calcium or magnesium. The safe concentration of methyl mercuric chloride for trout is about 1 μg/l (Drummond et al, 1974). Therefore, this method requires application factors to estimate safe concentrations.

Table 3

Metals that affect breathing patterns of fish.

Metal	Parameter	Fish	Minimum Effective Concentration (μg/l)	Exposure Time	Safe-unsafe Concentration Range (μg/l)	96 hour LC50 (μg/l)	Author
Zinc	Opercular breathing rate	bluegills	2550	< 24 h	76 - 235	≈ 10,000	Sparks et al, 1972
Copper Cadmium	Opercular breathing rate	largemouth bass	100 50	< 24 h < 24 h	? ?	100-1000 50-100	Morgan and Kuhn, 1974
Copper	Cough frequency	brook trout	9	< 24 h	9.5-17.4	110	Drummond *et al*, 1973
methyl mercuric chloride	Cough frequency	brook trout	3	8 days	1-3	?	Drummond *et al*, 1974

Table 4

Metals that affect blood chemistry

Metal	Parameter	Fish	Minimum Effective Concentration (µg/l)	Exposure Time	Safe-unsafe Concentration Range (µg/l)	96 hour LC50 (µg/l)	Author
methyl mercuric chloride	Hematocrit	rainbow trout	10	12 wks	1-3 *	?	O'Connor and Fromm, 1975
Copper	Plasma osmolarity	golden shiners channel catfish	2500	96 h	?	2,500-5000	Lewis and Lewis, 1971.
Zinc		golden shiners channel catfish	8000-12000	96 h	?	< 12000	
Copper	red blood cell counts	brook trout	24 - 37	6 and 21 days - no effect at 337 days	9.5-17.4	110	McKim et al, 1970
	hematocrit		"			"	
	hemoglobin		"			"	
	plasma chloride		"			"	
	plasma osmolarity		"			"	
	plasma protein		"			"	
Copper	red blood cell counts	brown bullheads	no effect after 600 days		?	180	Cristensen et al, 1972
	hematocrit		27 - 49	30 days**		"	
	hemoglobin		"	" **		"	
	plasma glucose		"	" **		"	
	plasma protein		no effect after 600 days			"	
	plasma chloride		27 - 49	30 days**		"	

** returned to normal after 600 days.

*Drummond et al, 1974 for brook trout.

Lewis and Lewis (1971) found that serum osmolarity of golden shiners and channel catfish was significantly depressed by 96-hour exposures to 2.5 mg/l Cu or 8-12 mg/l zinc. Since these concentrations are well within the lethal range, the test appears unpromising.

Significant changes in total RBC's, hematocrit, hemoglobin and plasma chloride, osmolarity and protein after 6 and 21 days exposure to copper suggested that 24 µg/l of copper was safe for brook trout (McKim et al, 1970). After 337 days, all values had returned to normal. An identical study by Christensen et al, (1972) estimated safe concentrations for brown bullheads (Ictalurus nebulosus) of 27 µg/l copper. Again, all values had returned to normal by the end of the experiment. Since the safe concentration of copper for brook trout is 9.5 µg/l, and since the responses are not constant, measurement of these parameters does not appear to provide a good short-cut method.,

Endocrinology

Sockeye salmon (Oncorhynchus nerka) exhibited a stress response to copper within 1-8 hours consisting of increased plasma corticosteroid concentrations (Donaldson and Dye, 1975). Significant increases occurred at 63.5 µg/l. Since this range encompasses the safe concentration for brook trout, the test should be re-evaluated using brook trout.

Enzyme activity

Enzyme activity may be a good indicator of metal effects on fish due to high sensitivity to inhibition by metals, specificity of some metal - enzyme reactions, ease of assay, etc. In addition, release to the plasma of enzymes such as glutamic oxalacetic transaminase is a good indicator of tissue damage (Christensen et al, 1972). To be of use, change in enzyme activity must be related to exposure to a metal. Consequently in vitro exposures of enzymes to metals may provide little useful information. While they may indicate which metals are most toxic to a given enzyme from a given tissue, the metals in vivo may never reach the enzyme. This information would only be of use if the authors related tissue concentrations of metals to degree of exposure of the animal and then tissue concentrations to enzyme inhibition. The problem is best illustrated by Jackim et al (1970) who compared activities of four liver enzymes of killifish after in vivo and in vitro exposures to six metals.

Their experiments (Table 5) demonstrated that:

1. inhibition of enzyme activity in vitro is not necessarily an indication of inhibition in vivo. Alkaline phosphatase activity in vitro was uniformly inhibited by the six metals, although to varying degrees. In vivo however, four metals had either no effect or increased activity.

2. The degree of sensitivity of the enzymes in vitro often did not reflect degree of sensitivity in vivo. In fact, the enzymes tested were remarkably insensitive to acute short-term in vivo exposures.

Although the enzymes assayed came from fish surviving 96 hour exposures to lethal concentrations of the metals, enzyme inhibition never exceeded 35%. Therefore, these enzymes do not appear as likely

Table 5

Effect of *in vivo* and *in vitro* exposures to metals on enzyme activity of killifish*
(Jackim *et al*, 1970)

		Alkaline Phosphatase			Acid phosphatase			Xanthine oxidase			Catalase		
		Decreased Activity	Increased Activity	No Effect	Decreased Activity	Increased Activity	No Effect	Decreased Activity	Increased Activity	No Effect	Decreased Activity	Increased Activity	No Effect
Beryllium	in vivo	+				Not tested			Not tested		Not tested		
	in vitro	++++											
Cadmium	in vivo			+	+			+			+		
	in vitro	++++			+					+	+++		
Copper	in vivo			+	+				++		+++		
	in vitro	++			+++			+++++			+		
Lead	in vivo		+		+++			+++			+++		
	in vitro	+			++++			++			++		
Mercury	in vivo		+		+			++++			++		
	in vitro	++			++			+++			+++		
Silver	in vivo	++					+	++			+++		
	in vitro	++			+++			++			++		

* In vivo measurements involved testing survivors after exposure to 96 hour LC50's.

short-term indicators despite their requirement for metals, their involvement in mineral metabolism (Jackim et al, 1970) or their in vitro sensitivity.

Because of the problems of interpreting in vitro exposures, I have not included the data of Christensen (1971, 1972) in this discussion.

Hinton and Koenig (1975) Table 6, demonstrated that acid phosphatase activities of subcellular fractions of channel catfish liver were inhibited 72 hours after an intraperitoneal injection of 15 mg/kg methyl mercuric chloride. This bioassay provides a means of assessing specific doses of toxicant. To make it a useful bioassay, dose injected should be correlated to dose received via food to give realistic dose size and frequency. Since the method did not demonstrate dramatic differences in activity, it might not be capable of identifying responses to lower dose rates.

Jackim (1973) measured inhibition of δ-amino levulinic acid dehydratase activity in livers and kidneys of mummichog and flounder after exposure in vivo to lead, mercury, copper, cadmium, zinc and silver. After two weeks, cadmium (10 mg/l), zinc (10 mg/l) and silver (10 mg/l) produced a slight enhancement of activity in mummichog while copper (1 mg/l) and lead (10 mg/l) caused a slight depression of activity. Lead at 50 mg/l and mercury at 0.02 mg/l caused 40-60% inhibition in livers of mummichogs exposed for 7-14 days. After 20 days, activity in mercury exposed fish returned to normal whereas it remained depressed in lead-exposed fish.

Table 6

Metals that affect enzyme activity of fish

Metal	Parameter	Fish	Minimum Effective Concentration (μg/l)	Exposure Time	Safe-unsafe Concentration Range (μg/l)	96 hour LC50 (μg/l)	Author
methyl mercuric chloride one dose intra peritoneally	Acid phosphatase in subcellular fractions of liver	channel catfish	15 mg/kg	72 h	?	?	Hinton and Koenig, 1975
Lead	Kidney and liver δ-aminolevulinic acid dehydratase	mummichog flounder	10,000	2 wks	?	188,000	Jackim, 1973
Mercury			20	7-14 days*	?	230	
Copper			1000	7-14 days*	?	3200	
Cadmium			no effect		?	27,000	
Zinc			no effect		?	40	
Silver			no effect		?	7	
Copper	Plasma glutamic oxalacetic transaminase	brook trout	24-39	6 and 21 days	9.5-17.4	110	McKim _et_ _al_, 1970
Copper			9.5-17.4	337 days	9.5-17.4	110	
Copper	Plasma glutamic oxalacetic transaminase	brown bullheads	27	600 days	?	180	Cristensen _et_ _al_, 1972
Copper	Plasma lactic dehydrogenase	brown bullheads	no effect	600 days	?	180	Cristensen _et_ _al_, 1972

*returned to normal in 20 days

At 10 mg/l lead, activity in liver tissue of flounder was reduced by 66% and by 58% in kidney.

These results demonstrate that lead is a fairly effective inhibitor of δ-amino levulinic acid dehydratase of liver and kidneys. However the results are difficult to interpret because

1) metal concentrations in water decreased continuously in the static exposure situation. This was complicated by the salt water bioassay.
2) the enzyme was assayed at 35 C while the fish were acclimated and exposed at 20 C. Since enzymes of poikilotherms may change biochemically in response to temperature changes (Hochachka and Somers, 1973) the activity in the presence of metals at 35 C may be totally different than at 20 C.
3) There are no data on safe concentrations of lead for mummichog and flounder. However, the 96 hour LC50 of lead for mummichog is 188 mg/l (NAS, 1974), a concentration much higher than those tested.

Plasma glutamic oxalacetic transaminase activity of brook trout plasma was elevated by exposure of the fish to more than 24 μg/l copper for 6 and 21 days (McKim et al, 1970). After 337 days, enzyme activity was depressed at concentrations greater than 9.5 μg/l. Therefore, the longer the exposure time, the lower the predicted safe concentration based on changes in enzyme activity. However, since the direction of the change varies, this method should be used with caution.

Christensen et al (1972) also assayed activity of plasma glutamic oxalacetic transaminase and lactic dehydrogenase of brown bullheads exposed to copper. Activity increased after 6 and 30 days at concentrations as low as 27 μg/l but changes were not significant. Significant decreases were observed only for PGOT at 27 μg/l or greater after 600 days of exposure. The results estimated a safe concentration for copper of 16-27 μg/l. However, the safe concentration for brown bullheads is unknown and the effect was observable only after very long exposure times.

Therefore, it is obvious that enzyme assays have not yet been used to great advantage for estimation of metal concentrations safe for fish. The following is an evaluation of erythrocyte δ-amino levulinic acid dehydratase (ALAD) activity of rainbow trout as an indicator of a harmful exposure to lead.

ALAD is an enzyme that catalyzes the condensation of two molecules of amino-levulinic acid to form porphobilinogen, a precursor of heme. Since porphobilinogen is required for the formation of hemoglobin, cytochromes, chlorophyll, vitamin B_{12}, etc. the enzyme system may be found in bacteria, algae, invertebrates and vertebrates. In animals having hemoglobin as an oxygen carrier, it is found in high concentrations in the blood. Since it is extremely sensitive to inhibition by lead, it is used to detect harmful lead exposures of humans.

The inhibition of the enzyme causes some anemia but the major effect of lead is on the nervous system. Therefore, the enzyme is an indicator of harmful lead uptake and may be useful for evaluating harmful lead exposure of a variety of organisms.

Materials and Methods

Rainbow trout blood was assayed for ALAD activity by a method modified from Granick et al (1972). The tail of each fish was severed and two samples of 75 μl of blood were collected from the caudal artery with chilled, heparinized, capillary tubes. One tube was capped and centrifuged to measure the microhematocrit. Blood from the second tube was immediately transferred to parafilm and 20 μl aliquots were added to each of three disposable centrifuge tubes at the animals acclimation temperature. The first was a blank and contained 100 μl of 0.2% Triton X-100 in 0.1 M, pH 6.2 phosphate buffer. The second and third tubes were duplicate tests and contained 100 μl of 0.2% Triton X-100 and 670 μg/l of amino levulinic acid in 0.1 M, pH 6.2 phosphate buffer.

The tubes were capped and the samples mixed and incubated at the acclimation temperature for two hours. The reaction was stopped with 300 μl of an aqueous solution of 4.0 g of trichloroacetic acid and 2.7 g of mercuric chloride per 100 ml. Each tube was centrifuged at 2500 x g for five minutes and 350 μl of supernatant was transferred to a glass test tube. The amount of porphobilinogen produced in the reaction was measured by adding 300 μl of modified Ehrlich's reagent to each test tube. This reagent consisted of 168 ml glacial acetic acid, 40 ml of 70% perchloric acid, 4.0 g of p-dimethyl amino benzaldehyde, 0.7 g mercuric chloride and 12 ml distilled water. The reagent was formulated immediately prior to each use.

After 15 minutes of colour development, absorbance of each test solution was read against the appropriate blank at 553 mμ using a spectrophotometer with micro cuvettes. ALAD activity, expressed as units/ml of red blood cells per hour, was given by the formula:

$$\text{Activity} = \frac{D \times A}{T \times H}$$

where D = dilution coefficient = 50
A = absorbance after two hours (average of two determinations)
T = incubation time (2 hours)
H = hematocrit $\div$ 100

Experimental designs were generally based on factorial or split-plot analysis of variance (Steel and Torrie, 1960) or a simple comparison of two treatments by a t-test. Experimental treatments were replicated a minimum of five times.

Results and Discussion

Two hours of ALAD activity in test solutions produced enough porphobilinogen for absorbance readings from 0.04 to 0.34. Most readings were between 0.1 and 0.2. Enzyme activity, calculated by the formula, ranged from 3.6 to 26.4 units per ml of red blood cells per hour. Activity of blood from control fish was usually greater than 20 units per ml of red blood cells per hour while that from exposed fish was less. When absolute values were transformed to a percentage of mean control concentrations, values ranged from 13 to 160%.

Using this method, we have first determined the pH optima for control trout and trout exposed to 100 μg/l lead for 1 week. Figure 1 demonstrates that the optimum pH is about 6.1 to 6.2. Lead has little effect on the pH optimum, in contrast to mammalian systems.

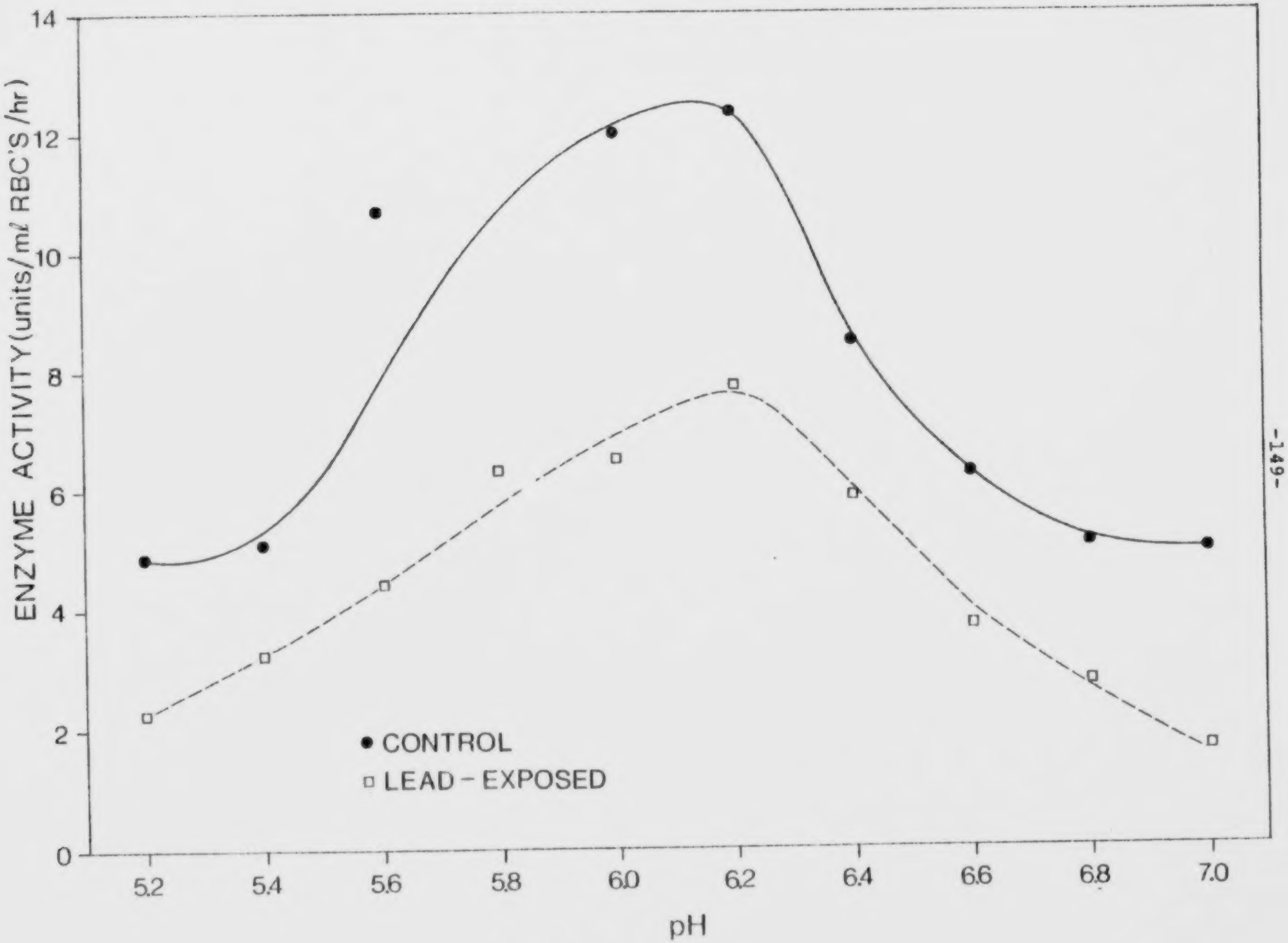

Figure 1 Variation with pH of erythrocyte ALAD activity of control rainbow trout and trout exposed to 600 μg/l lead for one week

The reaction was run at a substrate concentration allowing maximum velocity and the reaction rate was constant over the first 2 hours (Figure 2).

Amino levulinic acid dehydratase in fish blood is quite sensitive to lead exposure. Figure 3 indicates the activity in blood of rainbow trout and goldfish after 1 week exposure to a nominal 600 µg/l. Activity was inhibited by more than 60% in each case. This suggests that the enzyme will probably indicate exposure to lead in any fish species using hemoglobin as an oxygen carrier.

Other metals have little effect. Copper and cadmium, at concentrations just below the lethal level did not significantly change enzyme activity (Figure 4). Zinc stimulated the activity significantly but after 7 weeks of zinc exposure, activity was the same as control. Mercury at .05 mg/l, had no effect on activity after 2 weeks exposure.

When rainbow trout were exposed for 1 week to 0.6 mg/l lead, the activity of the enzyme decreased sharply to 35% of control (Figure 5). After transferral to clean water for two weeks, ALAD activity recovered to about 56% of control. Those fish remaining in lead solutions showed no recovery while control fish transferred to clean water maintained a high activity. These results suggest that a harmful lead exposure could be detected in fish several weeks after the cessation of exposure.

Figure 6 demonstrates that the degree of ALAD inhibition increases with lead concentration after a two week exposure. The lowest effect concentration appears to be about 18 µg/l.

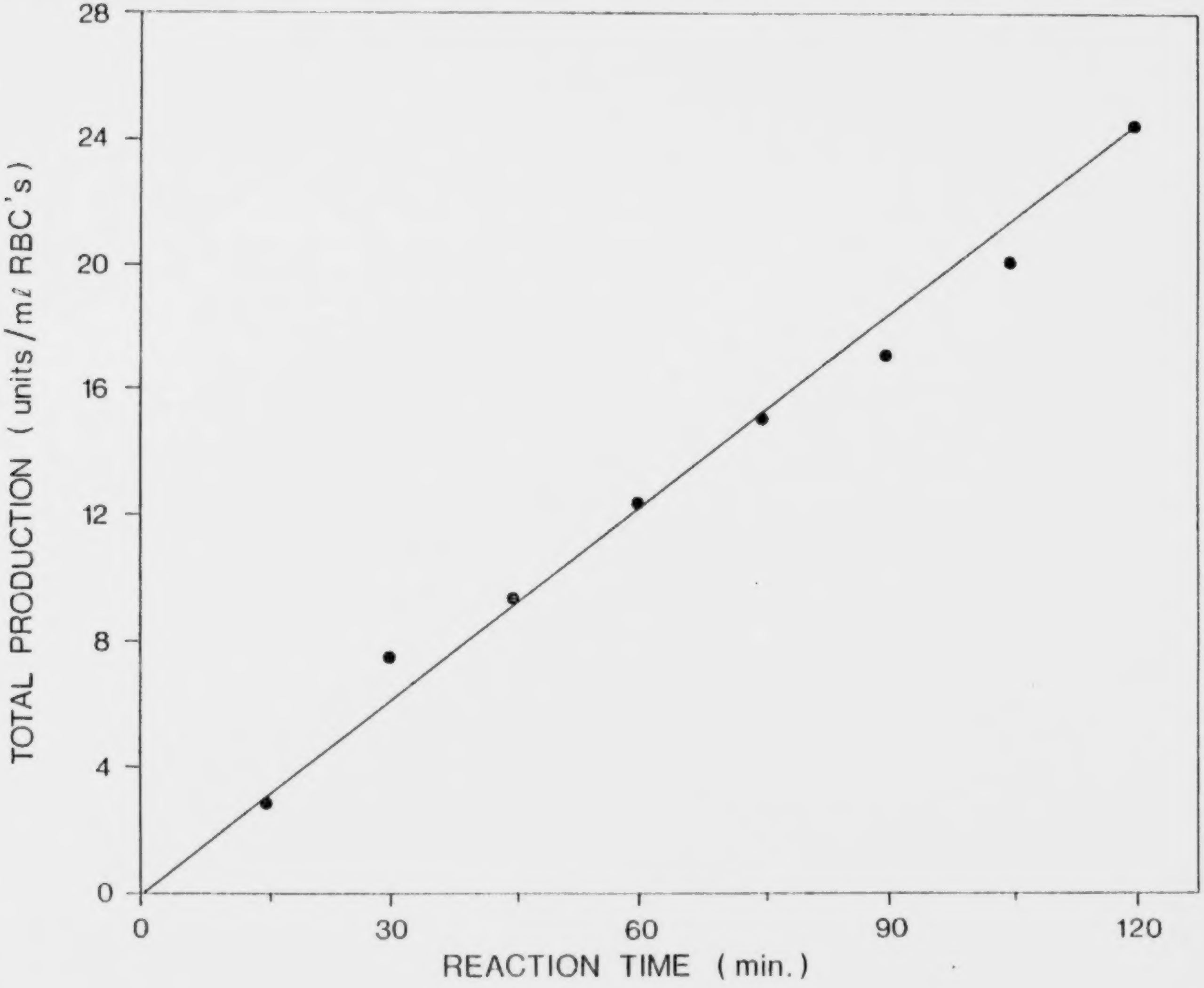

Figure 2 Variation with reaction time of porphobilinogen production by erythrocyte ALAD activity of rainbow trout.

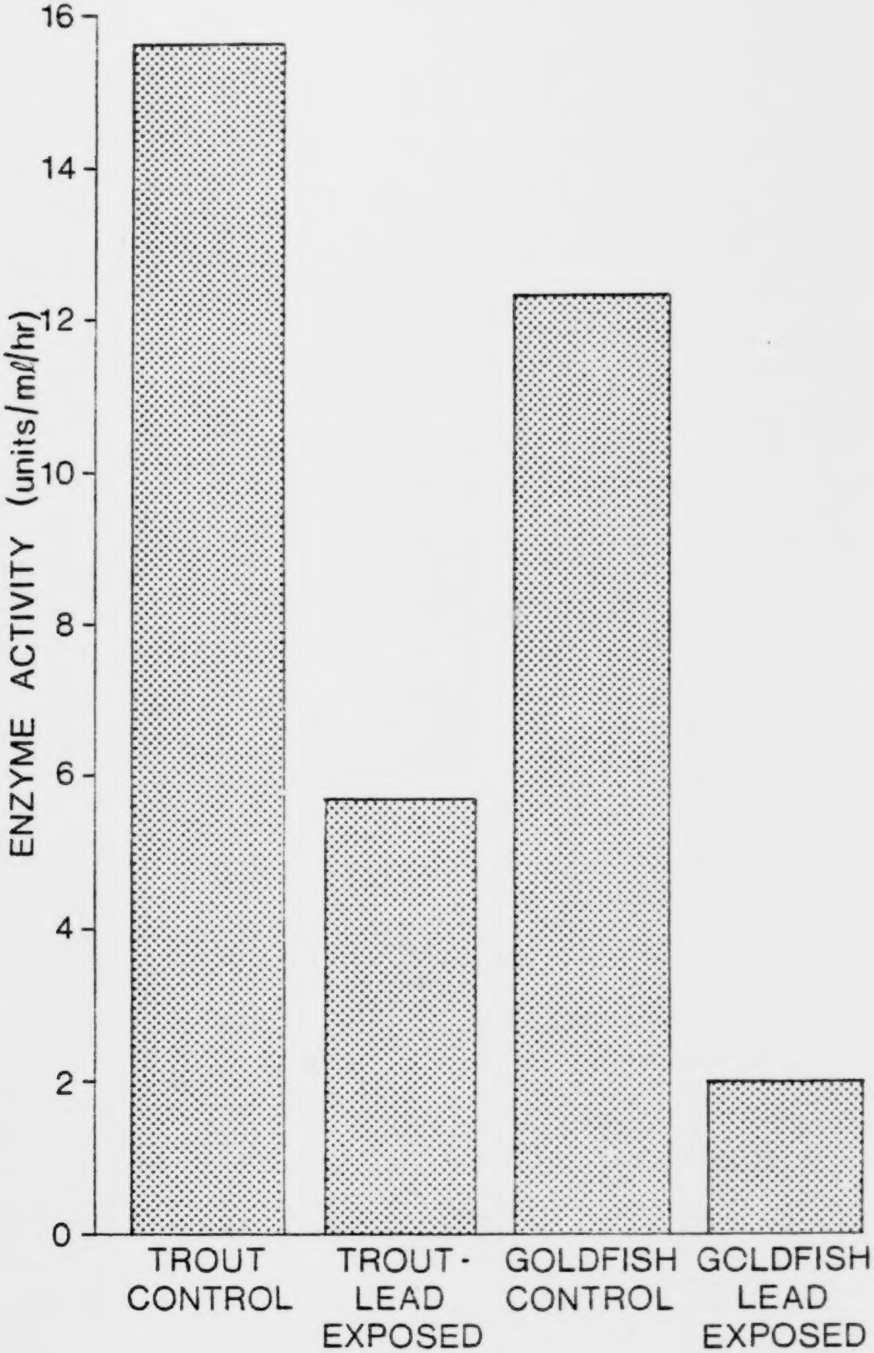

Figure 3 Erythrocyte ALAD activity of rainbow trout and goldfish exposed to no lead or 600 μg/l lead for one week.

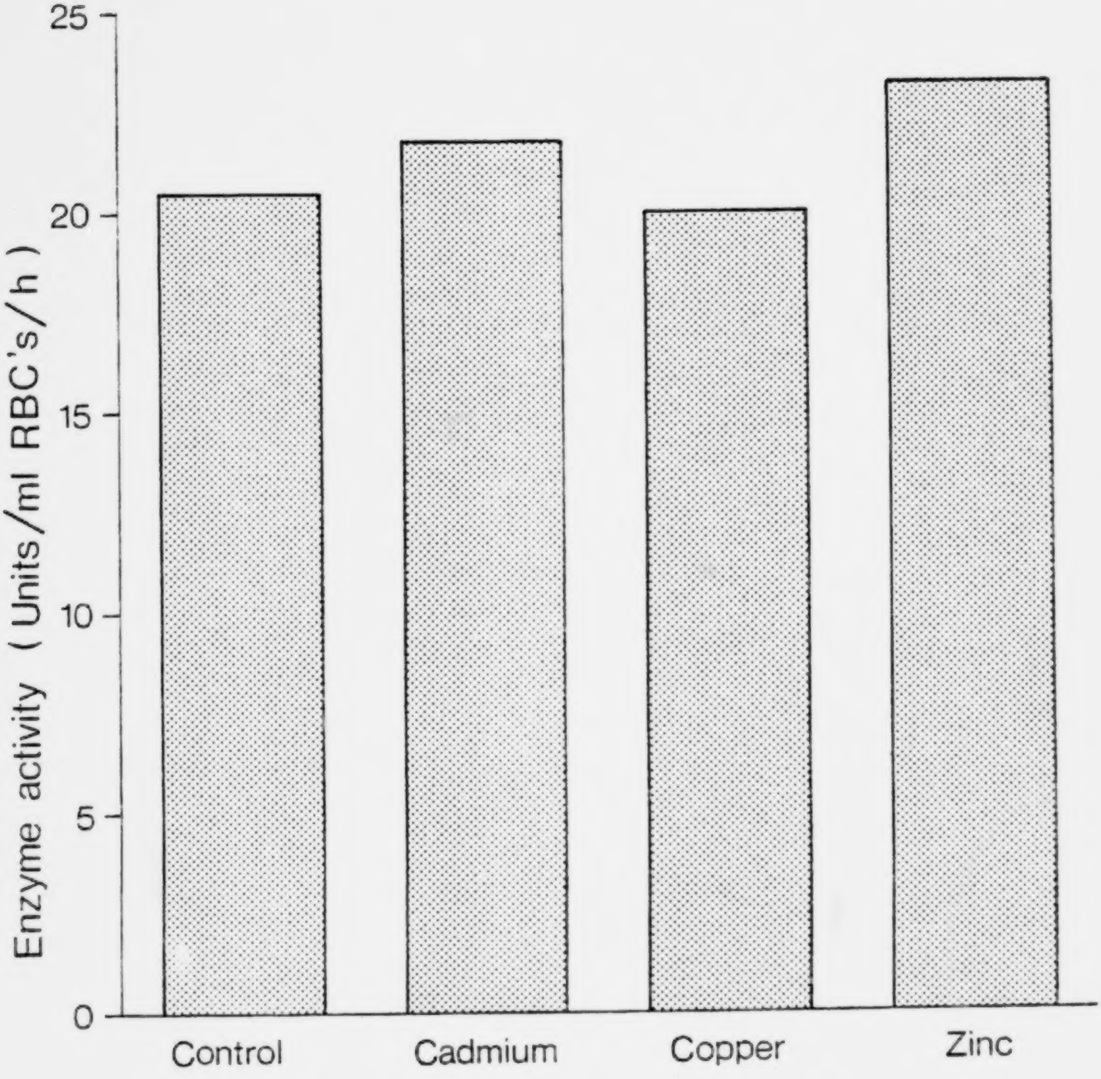

Figure 4 Erythrocyte ALAD activity of rainbow trout exposed to 50 µg/l cadmium, 100 µg/l copper and 100 µg/l

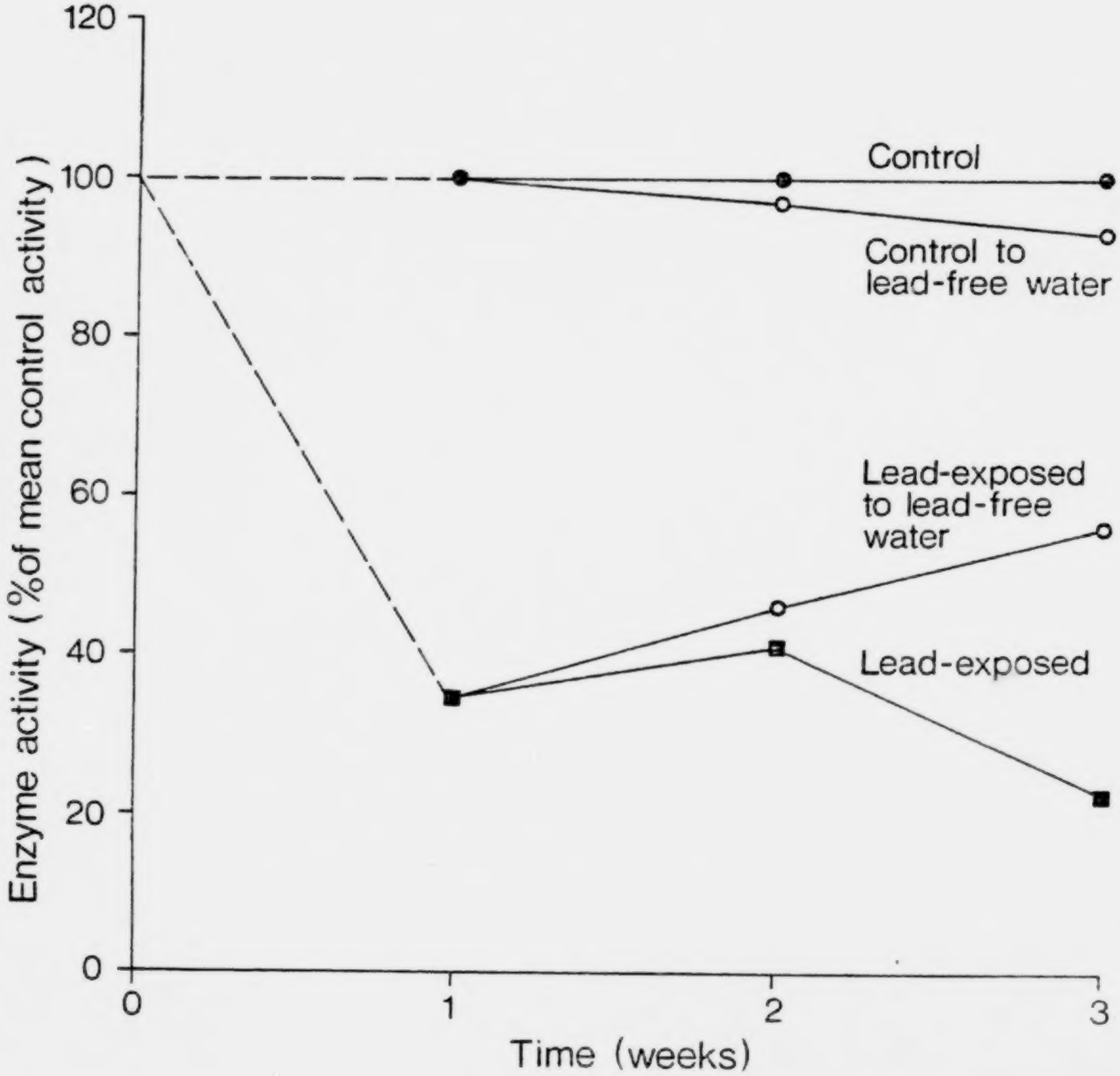

Figure 5 Recovery of erythrocyte ALAD activity of rainbow trout transferred to lead-free water after one week exposure to 600 μg/l lead.

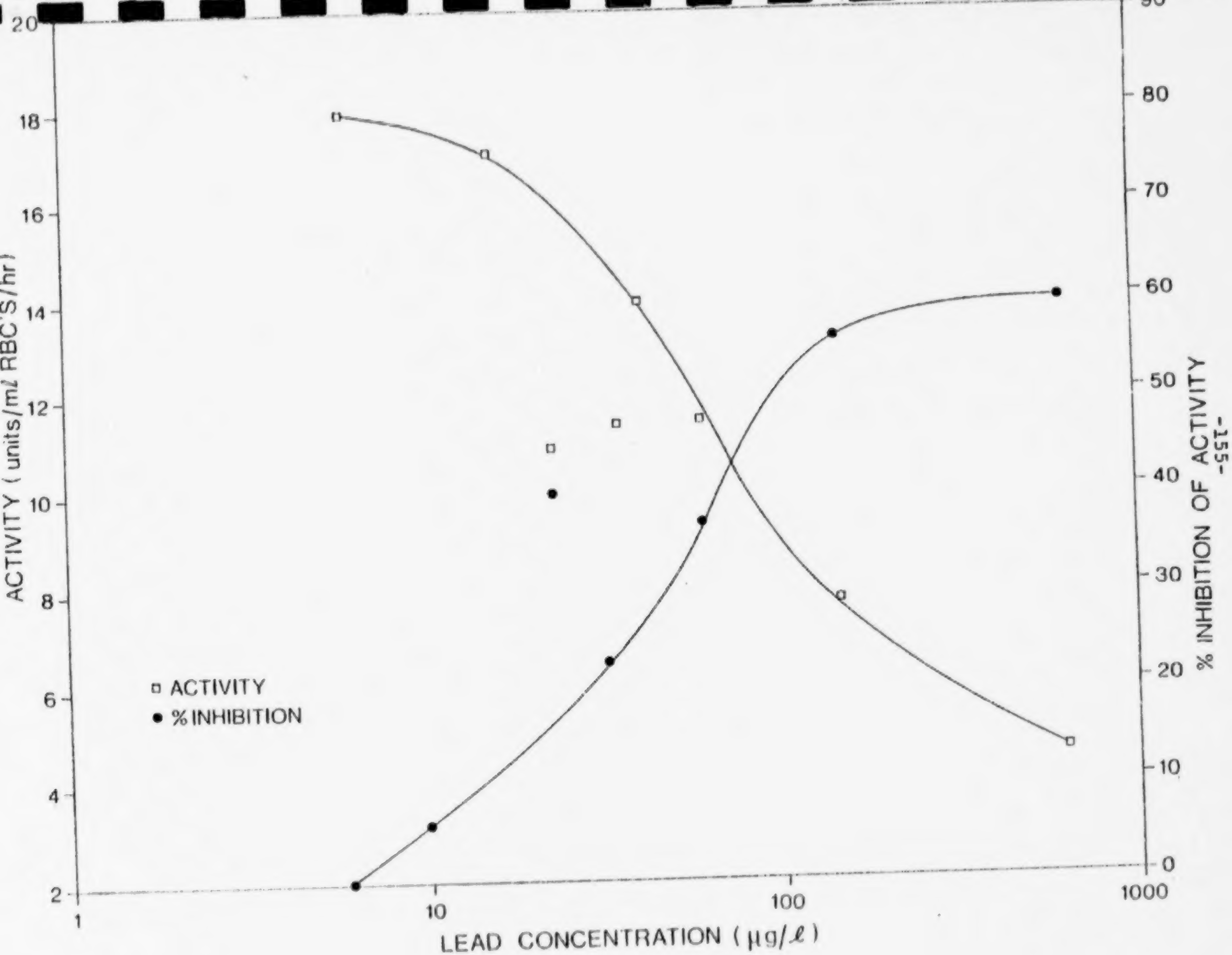

Figure 6 Effect of increasing concentrations of lead on erythrocyte ALAD activity of rainbow trout exposed to lead for two weeks. Percent inhibition is based on control activity.

When rainbow trout are exposed for increasing periods of time to lead, the degree of inhibition of ALAD increases. Figure 7 shows the results of a 16 week exposure to five measured concentrations of lead. The band across the slide indicates the minimum significant difference from control activity. After eight weeks, all concentrations of lead have caused significant inhibition of ALAD and the effect is even more apparent after 16 weeks.

Therefore, in fish, activity of red blood cell ALAD is depressed by exposure to lead in the water, the effect increasing both with lead concentration and exposure time. If ALAD activity is an index of a lead exposure harmful to rainbow trout, the data suggests that the safe concentration for lead lies between 3 and 13 μg/l lead.

Davies and Everhart (1973) measured chronic toxicity of lead to rainbow trout in soft and hard water. In soft water (26 mg/l alkalinity) the maximum acceptable toxicant concentration (MATC) was 6.0 to 11.9 g/l lead (Table 7). In hard water, (243 mg/l alkalinity), the MATC was 120 to 360 μg/l lead expressed as total lead, or 18 to 32 μg/l expressed as free lead. Extrapolating from these results, the MATC for free lead at an alkalinity of 90 mg/l is about 8 to 14 μg/l.

In this experiment, I observed no accumulation over time of precipitated lead as I have seen using higher lead concentrations. Therefore, I have assumed that all lead is in the free or dissolved form. ALAD inhibition, relative to controls, predicts an MATC of 3-13 μg/l for free lead. Since this range overlaps the range extrapolated from Davies and

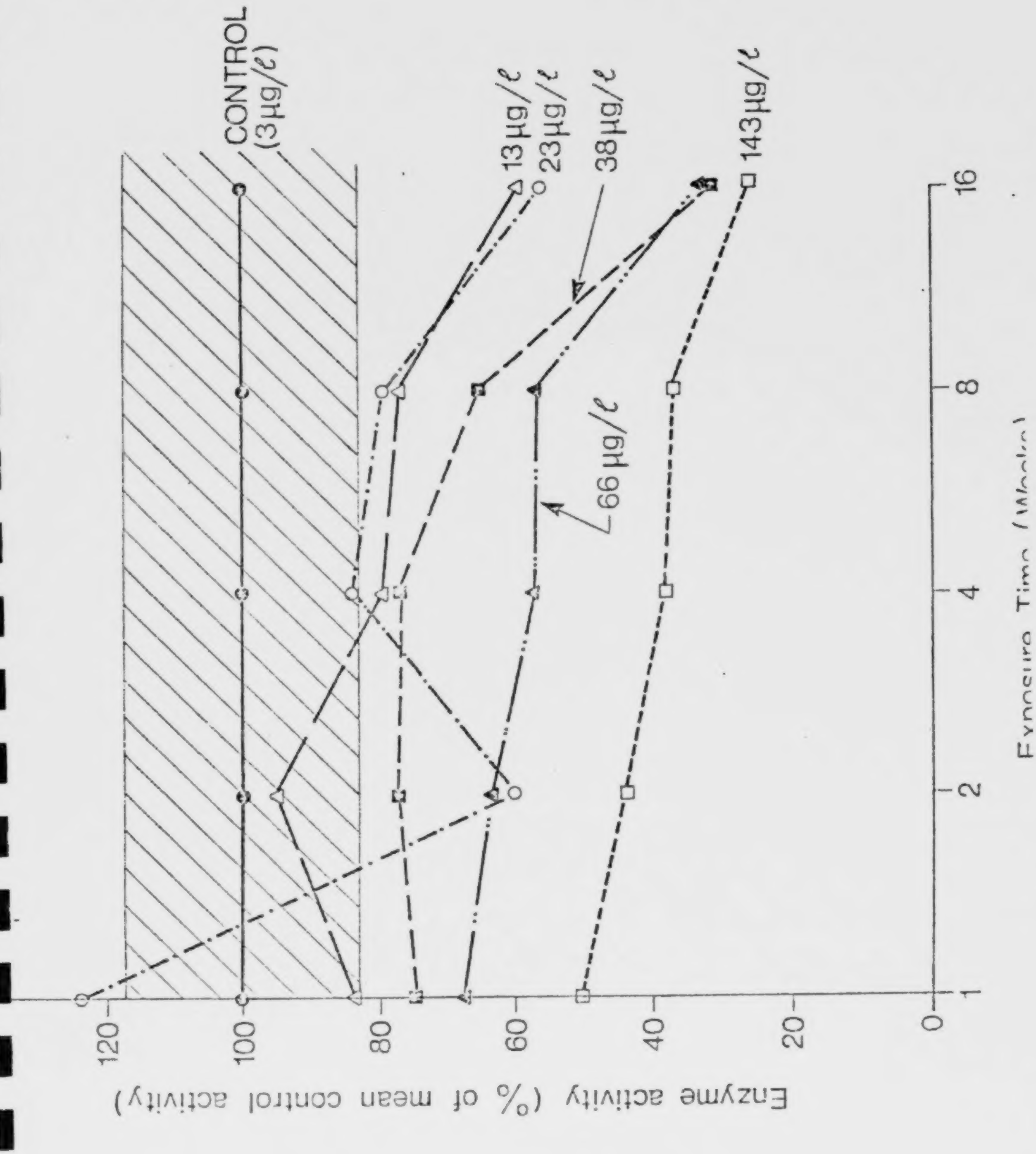

Figure 7 Effect of exposure time on inhibition of erythrocyte ALAD activity of trout exposed to six concentrations of lead. The hatched band indicates the minimum significant difference ($p < 0.05$) from control activity. Concentrations of lead are mean measured concentrations

Table 7

Summary of life cycle experiments designed to set a safe concentration for lead for trout.

(adapted from Davies and Everhart, 1973)

	Maximum no-effect concentration (= safe)		Minimum effect concentration (= unsafe)	
	Nominal (μg/l)	Measured by pulse polarography (μg/l)	Nominal (μg/l)	Measured by pulse polarography (μg/l)
Hard water (Hardness = 353 mg/l)	120	18	360	32
Soft water (Hardness = 28 mg/l)	6.2	6.0	12.5	11.9

Everhart's (1973) work, the measurement of enzyme activity appears to provide the same prediction of safe concentrations.

In summary, (Table 8) there are sublethal responses of fish to some metals appropriate for estimating safe concentrations for these metals. For most metals, use of specific responses will require further investigation before application to the problems of setting objectives. For arsenic, cadmium, chromium, nickel, selenium and silver, we have no short-cut methods. In fact, for arsenic, chromium, selenium and silver there is little published evidence for sublethal effects on fish.

Short-term indicators of long-term effects of metals on fish

Metal	Indicator	Parameter	Comment
Arsenic	--	--	--
Cadmium	--	--	--
Copper	Behaviour	Locomotor activity	
		Feeding activity	
		Avoidance	Not yet related to safe concentrations
	Breathing patterns	Cough frequency	
Chromium	--	--	--
Nickel	--	--	--
Lead	Enzymes	Erythrocyte δ-amino levulinic acid dehydratase activity	
Mercury	Behaviour	Learning	Not yet related to safe concentrations
Methyl mercuric chloride	Metabolism	Gill O_2 consumption	Requires small application factor
	Blood Chemistry	Hematocrit	"
	Breathing pattern	Cough frequency	
Selenium	--	--	--
Silver	--	--	--
Zinc	Behaviour	Avoidance	Not yet related to safe concentration.

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An Acute Bioassay for Cardiovascular and Respiratory Functions in Rainbow Trout

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Sub-lethal concentrations of toxicants can produce harmful effects on life-sustaining physiological functions, which could produce chronic, detrimental effects on fish populations. For example, toxicants producing a stress on cardiovascular/respiratory function may also affect the fish's ability to reproduce, to capture food, or to escape from predators. Several investigators, using a variety of methods, have demonstrated that parameters such as respiration rate, cough response, and heart rate are sensitive to chemicals.

For the past six years fenitrothion has been used in place of DDT as a means of spruce budworm control in Canadian forests. Recently acephate has been considered for this purpose, as investigators have shown that it is about as effective as fenitrothion for control of the spruce budworm Since the spraying of our forests may adversely affect the aquatic environment, the toxicity of fenitrothion and acephate to fish must be considered.

One objective of our study was to develop a bioassay that was short, technically simple, and sensitive to sub-lethal changes on the cardiovascular/respiratory systems of rainbow trout. Another objective was to investigate the sub-lethal effects produced on these systems by the two insecticides.

Forty-three rainbow trout of either sex with a mean (± SD) weight of 905 ± 197 gms and mean (± SD) length of 39.5 ± 3.4 cms were used in this study. These fish were held in thiosulphate dechlorinated city of Winnipeg tap water at 10°C for at least 1 month prior to this study.

At 9:00 A.M. on the morning of an experiment a fish was anesthetized in water containing 0.33 $m\ell/\ell$ of 2-phenoxyethanol. Catheters consisting of PE60 (Clay-Adams) tubing were implanted for the purpose of obtaining buccal and opercular amplitudes, and to obtain respiratory rate. Following this, electrocardiogram electrodes were inserted under the skin, and sewn in place. These procedures took less than 30 minutes, after which the fish were placed into a plexiglass restraining chamber. The volume of the chamber was 14.5 ℓ and the flow rate was 5.0 ℓ/min for the first 2 hrs. Test temperature was 10°C; DO was maintained at near saturation level by the use of an air stone within the chamber; and photoperiod was 12 hrs light, 12 hrs dark. After the first 2 hr equilibration period a toxicant delivery system located above the chamber was used to provide an intermittent flow of 1.0 ℓ/min. The fish was allowed to acclimate to this flow rate for one hour before any toxicant was added. After introducing the toxicant, the flow rate was maintained at 1.0 ℓ/min and the 99% replacement time was 1.1 hrs. Checks were performed hourly for the first 5 hrs., and then again at 9, 21, and 24 hrs. A four channel physiological recorder (Hewlett Packard #7754A) was used to record the ECG, as well as the opercular and buccal waveforms, which were obtained by the use of the 2 respiratory catheters attached to two pressure transducers (HP #1280C-02), in series with a pair of pressure amplifiers (HP #8805C). The ECG was obtained by the use of a bioelectric amplifier (HP #8811A).

From these waveforms and ECG data, heart and respiratory rates, as well as buccal and opercular amplitudes can be determined. From the respiratory traces cough responses can also be detected.

Before we were able to proceed with the fenitrothion study, it was necessary to find a suitable solvent for this insecticide. Acephate didn't present a problem, as it is more water soluble. The two solvents investigated were propylene glycol and acetone.

Data in the Figures are expressed as $\overline{X} \pm$ SE. Statistical analyses were performed using the "student's t" test, and results were considered significantly different if $p \leq 0.05$.

Figure 1 shows respiration rate in beats/min. plotted against time. Fish exposed to 3900 mg/ℓ of propylene glycol showed a slight, but significant decrease in rate from control values for the first hour. There was no significant difference for the remainder of the 24 hr. period. However, fish subjected to 2950 mg/ℓ of acetone showed a marked increase in rate within the first half hour, and the rate remained elevated throughout the course of the experiment. Figure 2 shows an elevated buccal amplitude after 9 hrs. of exposure to acetone, whereas there was no significant difference in amplitude between propylene glycol-exposed fish and controls.

On the basis of these data, and the fact that none of the other parameters tested were affected by propylene glycol, it appears that it is better suited for solubilizing fenitrothion than is acetone.

Figure 3 shows a dose-related response of coughs/min. vs. time. Fish exposed to 0.5 mg/ℓ fenitrothion showed a significant increase in coughs for the first 10 hrs. of our experiments, after which the coughs declined to control levels. Fish exposed to 1.0 and 5.0 mg/ℓ fenitrothion showed an immediate increase in coughing after exposure, and the rates in both concentrations remained elevated significantly over the 24 hr. exposure period.

Figure 4 shows the mean number of coughs/min. over the entire exposure period for each of the 3 concentrations of fenitrothion, plus propylene glycol. There was no difference between propylene glycol and control values, and the rate of coughs/min. was dependent on the concentration of fenitrothion.

Figure 5 shows that fish exposed to the relatively high concentration of 5.0 mg/ℓ fenitrothion showed a significant drop in heart rate immediately after exposure. Heart rate gradually returned to control values over the 24 hr. period. However, 3 of the fish were dead after 20 hrs. of exposure to this concentration. This can be explained by the fact that this concentration approaches the 24 hr. LC_{50} level for fingerling rainbow. Fish exposed to the lower concentrations of fenitrothion did not show this decrease in heart rate.

Figure 6 shows that fish exposed to 5.0 mg/ℓ fenitrothion showed a significant increase in the Q-T interval representing the time it took for the ventricle to depolarize, and then repolarize. This increase was significantly different only for the first hour of exposure.

In all 3 concentrations of fenitrothion, the heart did not beat in a regular sequence. This arrythmia occurred much more often at 5.0 mg/ℓ than it did at the lower concentrations, and it persisted throughout the entire exposure period at the high concentrations. Acephate-exposed fish also showed these irregularities in both 500 and 1,000 mg/ℓ concentrations but less frequently than did the fenitrothion-exposed fish.

Figure 7 presents heart rate data for fish exposed to 2 concentrations of acephate. It can be seen that at the 1,000 mg/ℓ level, heart rate decreased significantly after 1 hr. of exposure, and at 4, 21 and 24 hrs.

Figure 8 shows that at 1,000 mg/ℓ acephate, the Q-T intervals were significantly longer at 1, 3, 21 and 24 hrs. Neither heart rate nor the Q-T intervals were affected at 500 mg/ℓ acephate.

In conclusion, this bioassay has demonstrated that it can qualitatively and quantitatively define cardiovascular/respiratory physiological responses of fish to a variety of chemicals. For example, fish exposed to acetone showed an increase in respiration rate and amplitude. These responses are usually associated with an increase in the volume pumped across the gills. Acetone, when taken in by the mammalian system, produces an acid condition in the blood. The reflex to systemic acidosis is to hyperventilate to drive off excess CO_2. Possibly the increased rate and amplitude in fish is due to a similar reflex.

A dose-related cough response was elicited in fish exposed to 3 concentrations of fenitrothion. The cough response in fish is thought to be a gill clearing mechanism, reflecting the possibility that fenitrothion interferes with gill function in some way.

Fish exposed to 1,000 mg/ℓ acephate and 5.0 mg/ℓ fenitrothion showed a significant decrease in heart rate. In the case of acephate the decreased heart rate was accompanied by a prolonged Q-T interval, whereas with fenitrothion cardiac arrythmias played a more prominent role in lowering the heart rate. These arrythmias were also observed in fish exposed to lower concentrations of both these insecticides, but to a much lesser extent. Both acephate and fenitrothion are cholinesterase inhibitors, and the decrease in heart rate may be related to this fact, as the heart of rainbow trout is controlled by the vagus nerve, which is cholinergic.

Further work is needed to come to any definite conclusions about the mechanisms of toxicity of the chemicals used in this study. However, this bioassay system has proven to be a useful tool in monitoring physiological responses of fish to sub-lethal concentrations of chemicals.

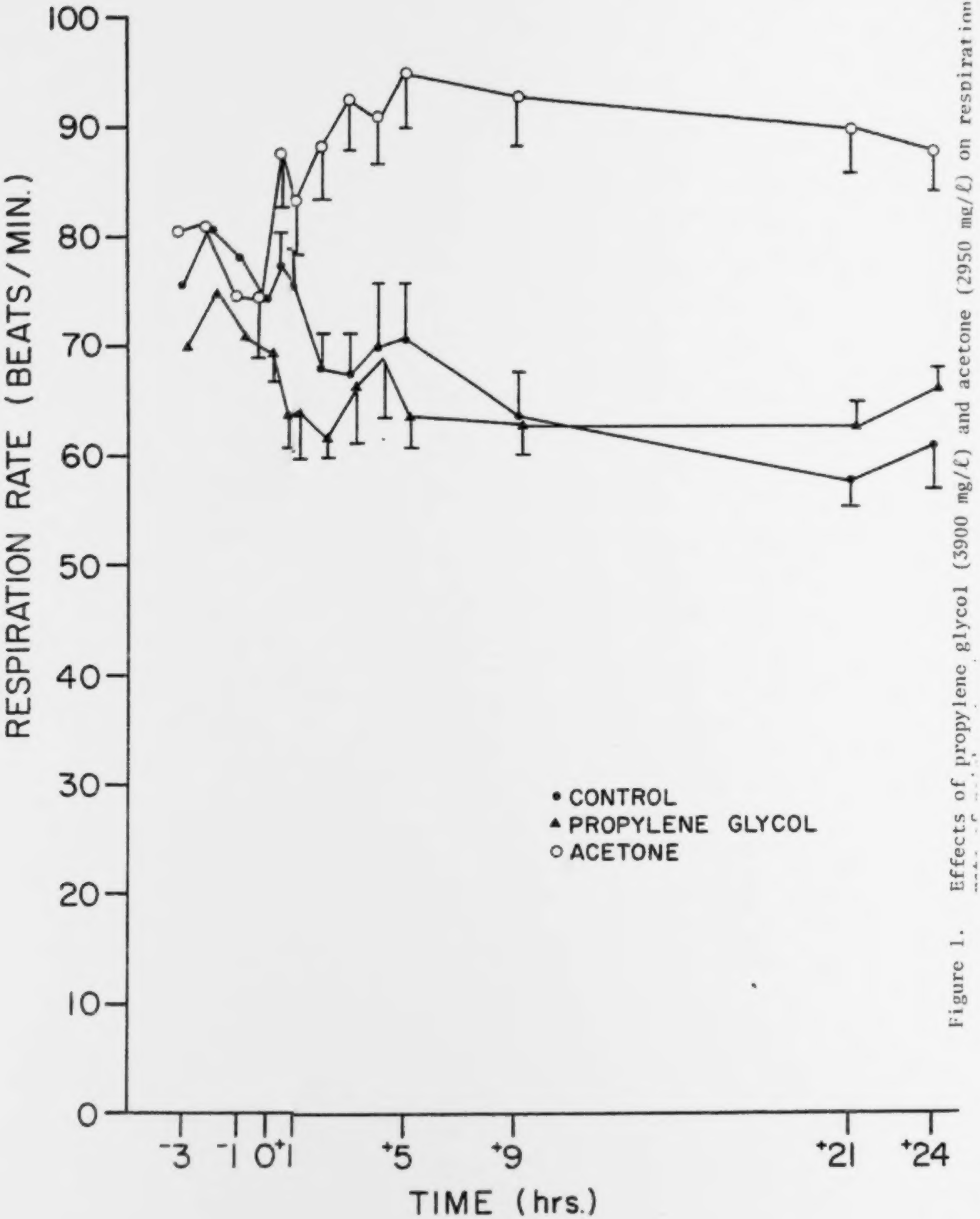

Figure 1. Effects of propylene glycol (3900 mg/ℓ) and acetone (2950 mg/ℓ) on respiration

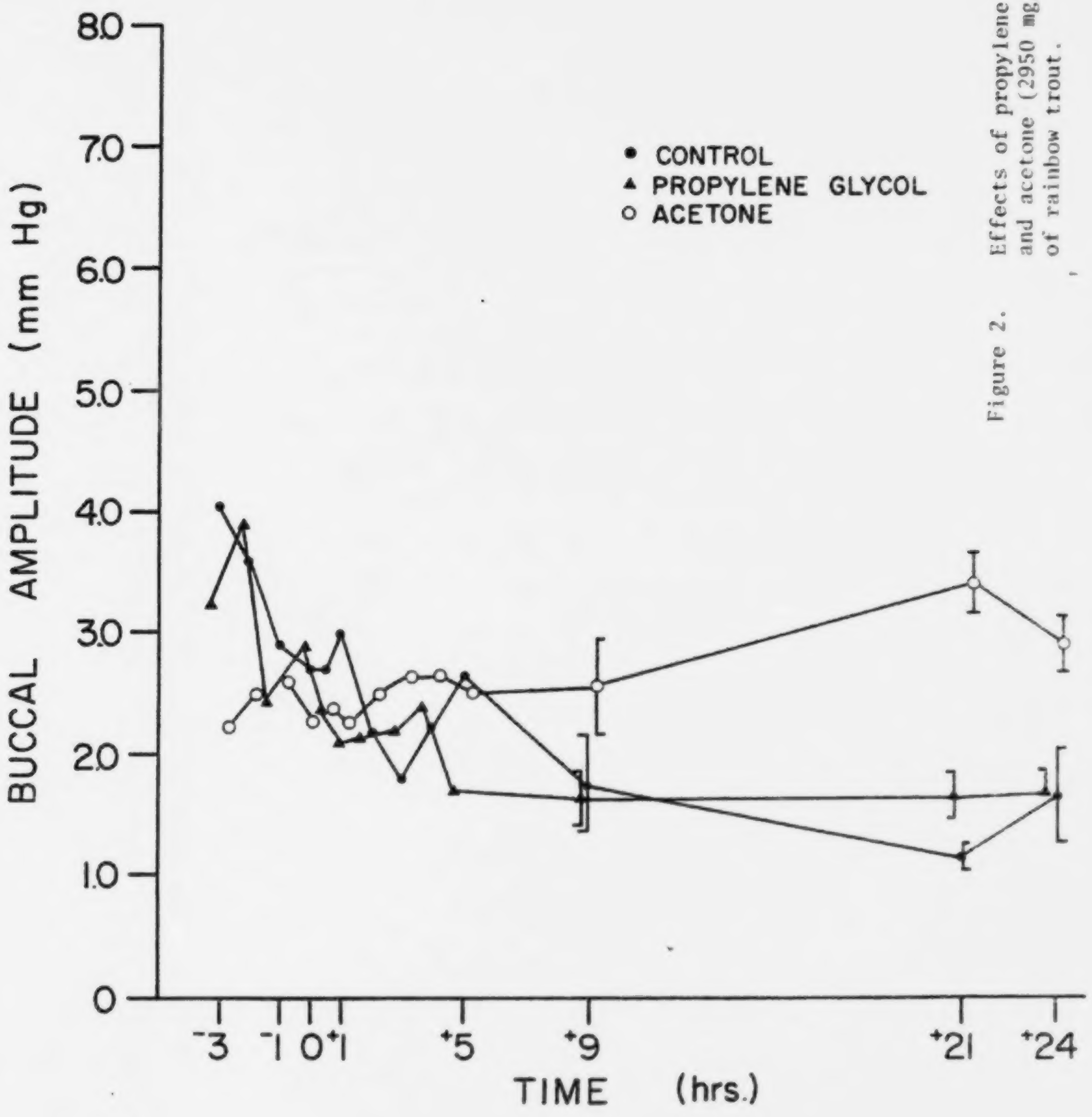

Figure 2. Effects of propylene glycol (3900 mg/ℓ) and and acetone (2950 mg/ℓ) on buccal amplitude of rainbow trout.

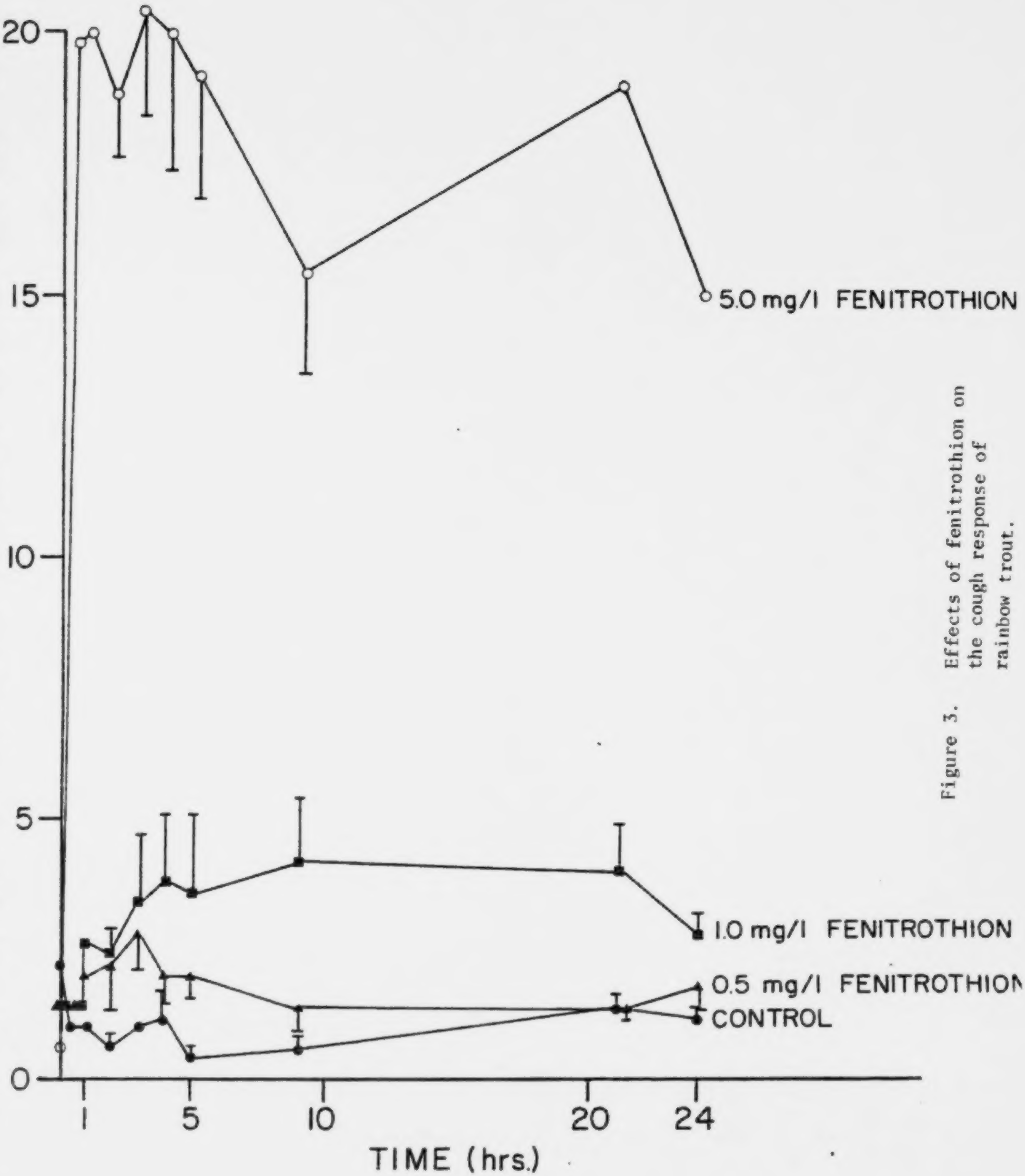

Figure 3. Effects of fenitrothion on the cough response of rainbow trout.

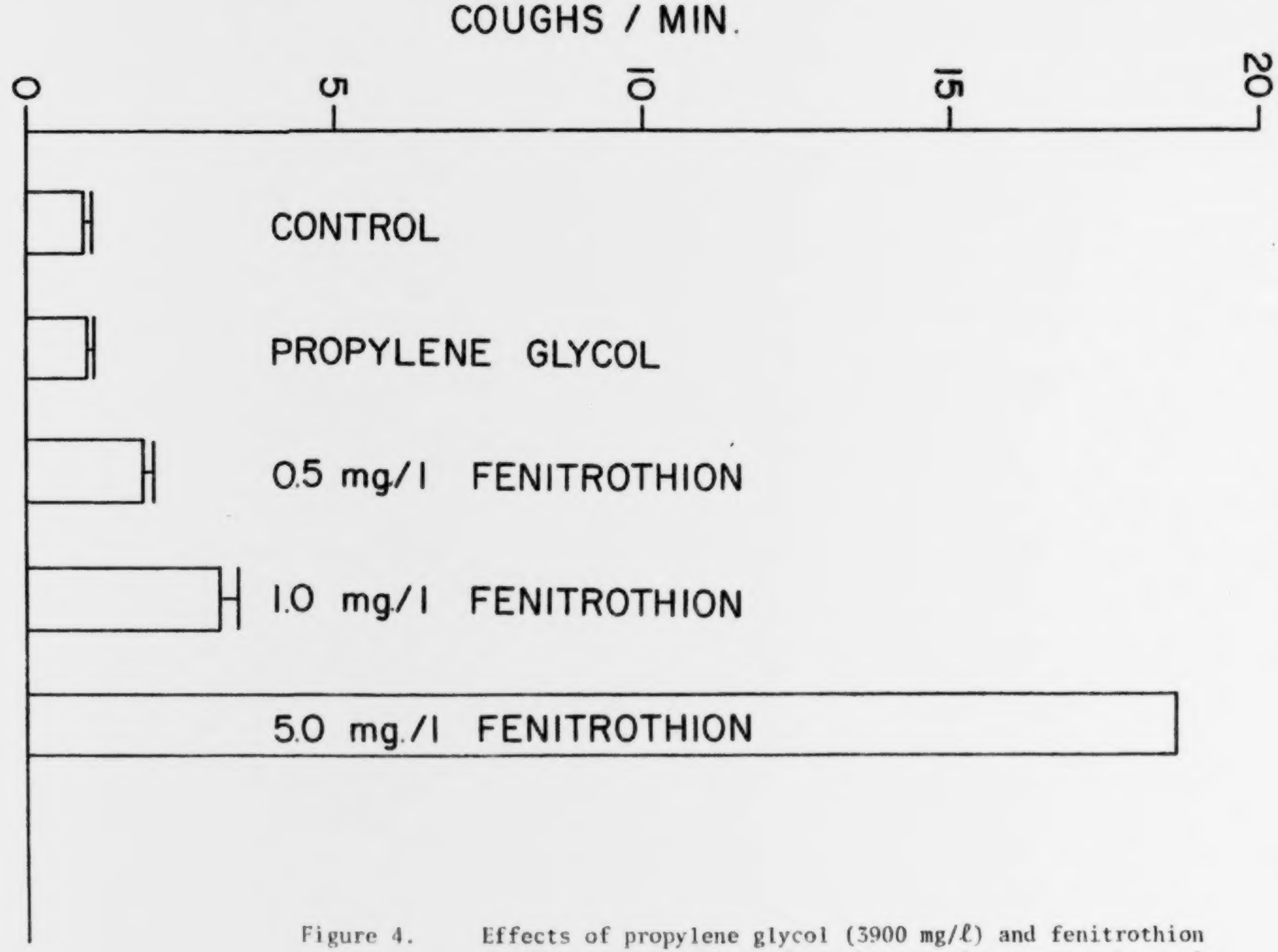

Figure 4. Effects of propylene glycol (3900 mg/ℓ) and fenitrothion on the average number of coughs per minute over the entire exposure period.

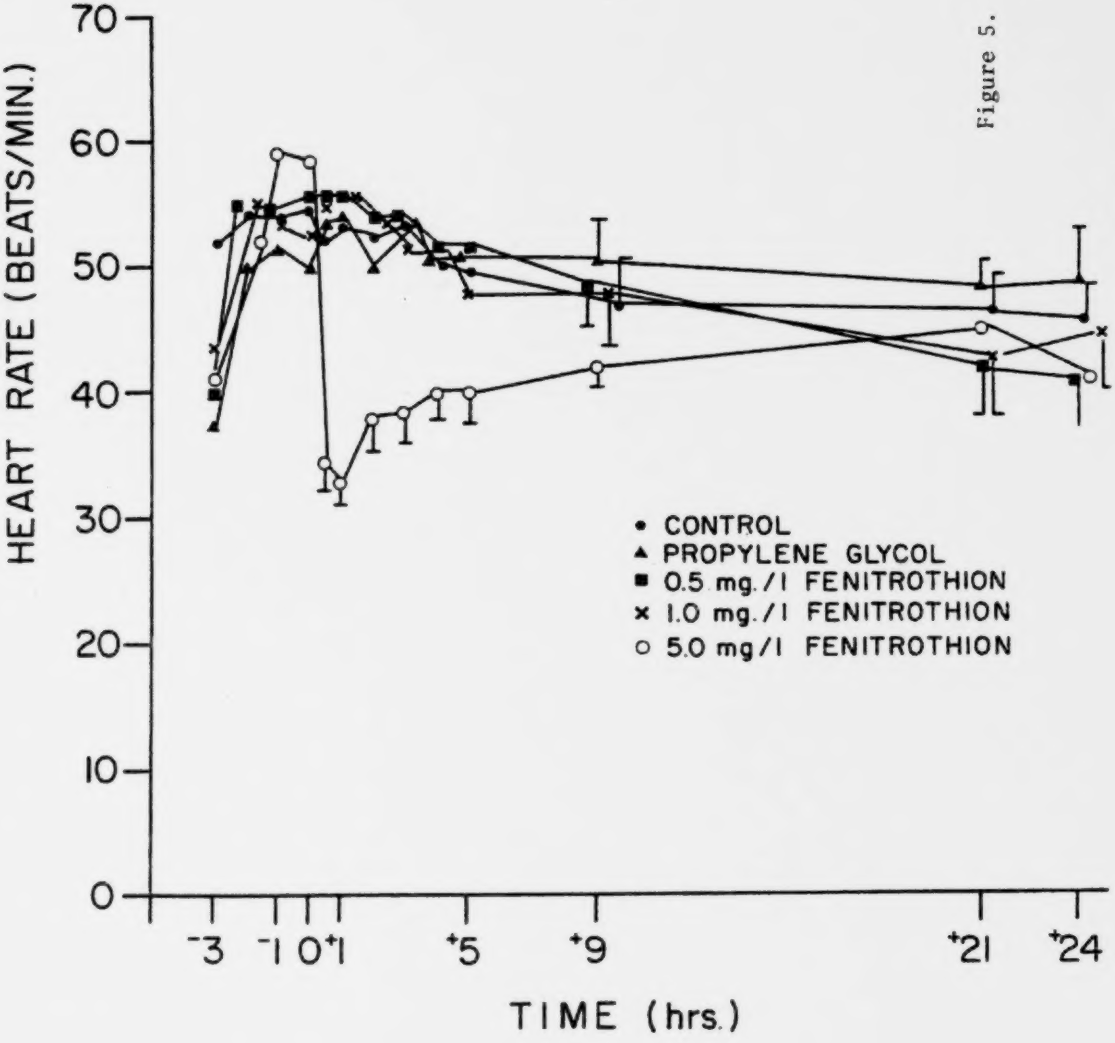

Figure 5. Effects of propylene glycol (3900 mg/ℓ) and fenitrothion on heart rate of rainbow trout.

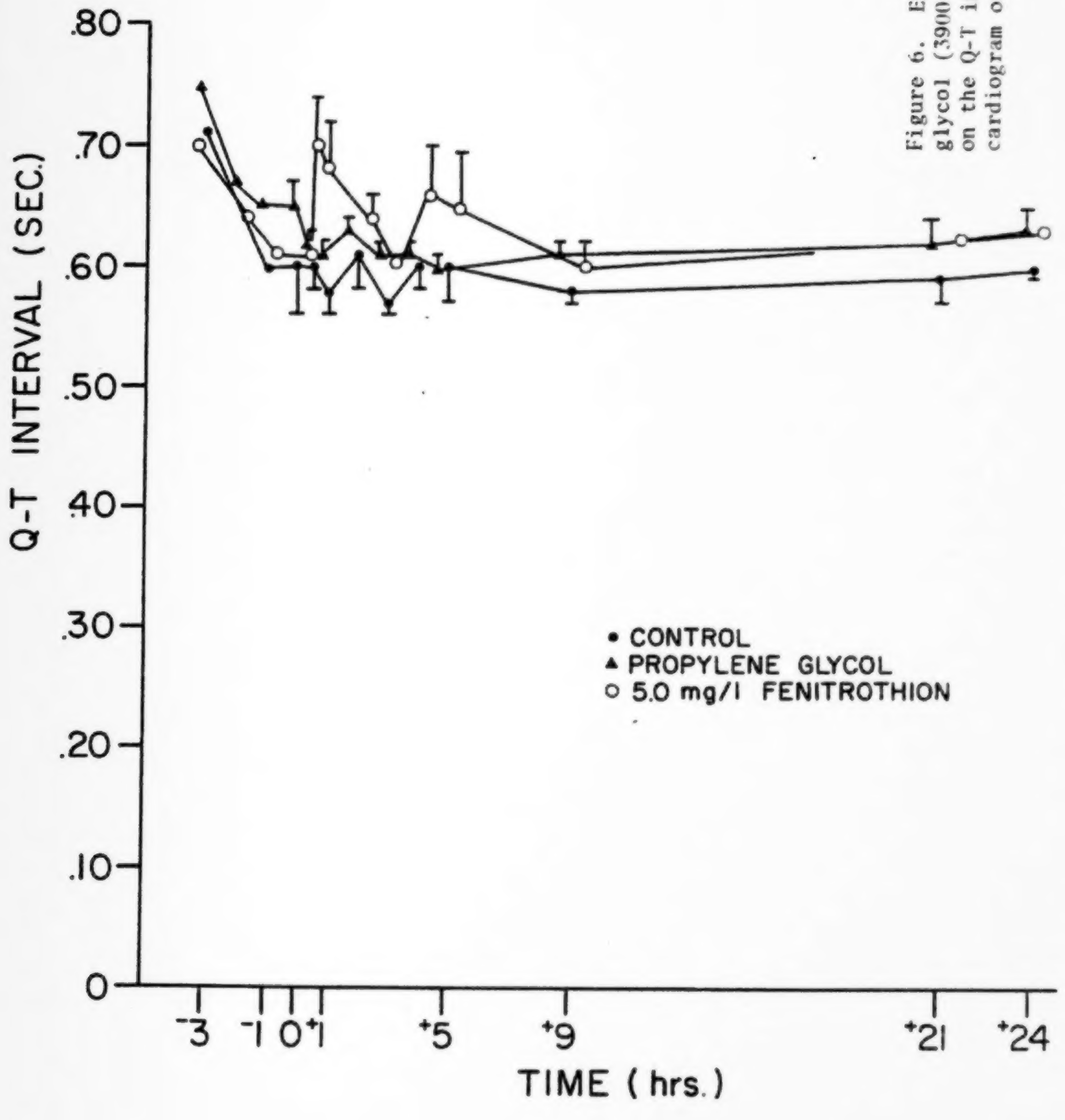

Figure 6. Effects of propylene glycol (3900 mg/ℓ) and fenitrothion on the Q-T interval of the electrocardiogram of rainbow trout.

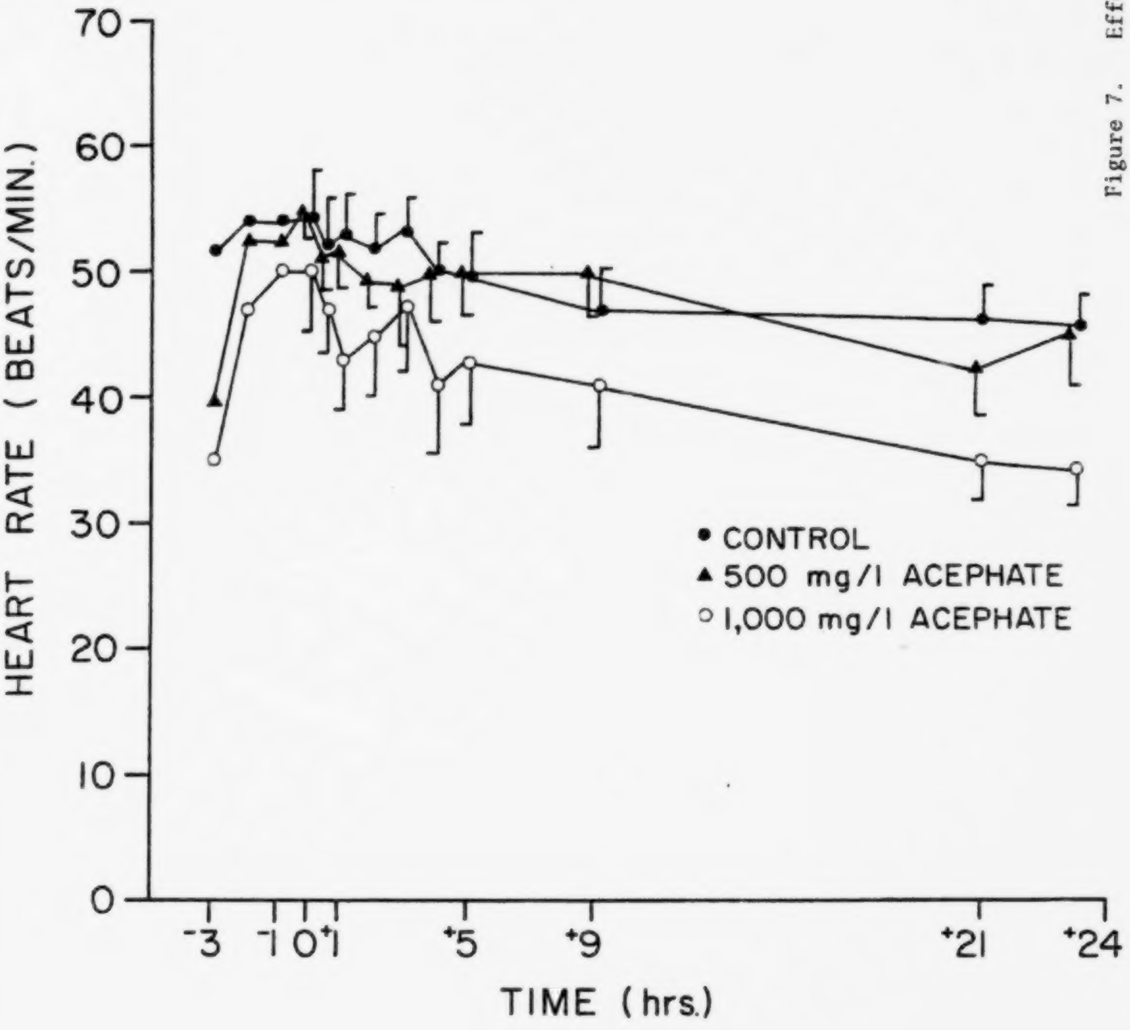

Figure 7. Effects of acephate on heart rate of rainbow trout.

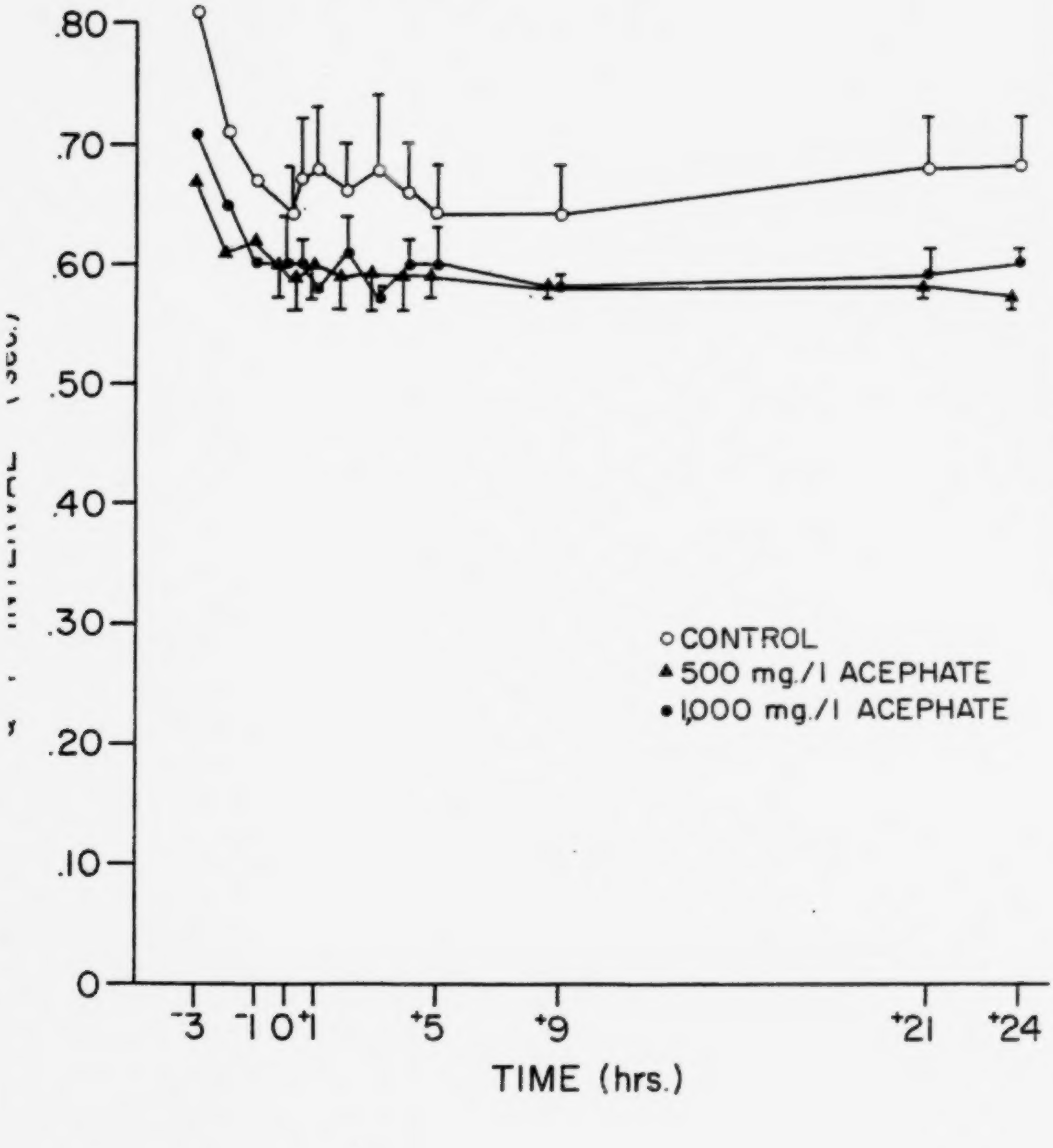

Figure 8. Effects of acephate on the Q-T interval of the electrocardiogram of rainbow trout.

AVOIDANCE: PREFERENCE RESPONSES
OF WHITEFISH TO MINE EFFLUENTS

by

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Avoidance: Preference Responses of Whitefish to Mine Effluents

Ken Supeene
Gary Alexander

INTRODUCTION

Avoidance: preference studies have been developed as one method to investigate the sublethal responses of fish to a toxicant. (Sprague and Saunders, 1963; Lawrence and Scherer, 1974). The basic aim of this sublethal method is to determine whether a toxicant will attract or repel a fish. This attraction or repulsion can become extremely significant if the pollutant is deleterious to the fish during chronic exposure. If a fish does not avoid the polluted water it may remain exposed to the toxicant, resulting in death or sublethal effects.

The avoidance: preference study described in this paper was one segment of a larger program involving acute and sublethal testing of Manitoba mine effluents conducted at the Freshwater Iestitute in Winnipeg. Mines A, B, and D discharged tailings into river-lake systems, while Mine C tailings were mixed with smelting effluent in a lake which subsequently entered a river.

MATERIALS AND METHODS

Mine effluents were collected between June and September of 1975. They were transported to Winnipeg and stored at 4°C. Mine B was neutralized to pH 6.8 from ≈ pH 11.0. The other mine effluents required no pH modification. Testing the avoidance: preference response of whitefish (Coregonus clupeaformis) required two weeks, including an initial determination of the 96 hr. LC_{50} for whitefish exposed to mine effluents.

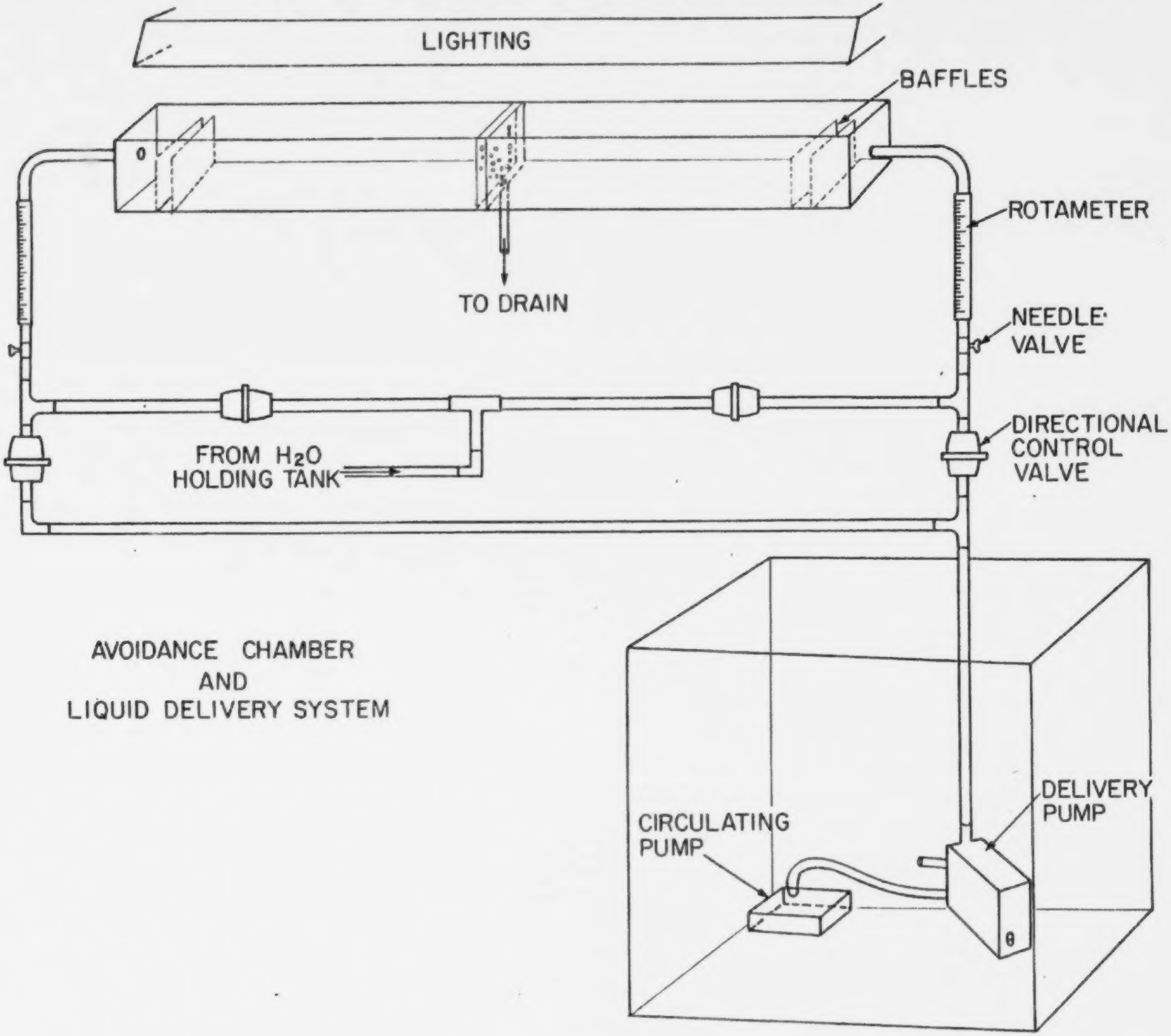
Whitefish ranging from 12-14 months with fork lengths of 7.3 - 11.7 cm were obtained from Steeprock Lake, Manitoba. Fish were acclimated at 15.0°C to U.V. dechlorinated Winnipeg city tap water for one month prior to testing.

A modified version of the avoidance system described by Scherer and Nowak (1973) was used in this study. The basic system involved the introduction of water from one end of a chamber and effluent from the other. The two flows were controlled to constant and equal rates by rotameters and adjustable needle valves.

A pair of baffles at each end of the chamber fully mixed the effluent and ensured a laminar flow entering the chamber. The liquids flowed towards the centre of the chamber where a constant interface between the liquids was formed by a series of three drains on each side.

A few notable modifications were made to the basic avoidance chamber water delivery system. There were the following:

1. The plumbing system was adjusted to enable delivery of either pure water or a premixed concentration of effluent to either side of the avoidance chamber.


LIGHTING
BAFFLES
ROTAMETER
TO DRAIN
NEEDLE VALVE
FROM H_2O HOLDING TANK
DIRECTIONAL CONTROL VALVE
DELIVERY PUMP
CIRCULATING PUMP

AVOIDANCE CHAMBER
AND
LIQUID DELIVERY SYSTEM

2. The effluent was held in a 60 gallon (273 ℓ) temperature controlled polyethylene reservoir. This volume was sufficient for five test replicates. A submersible pump mixed the effluent and another delivered the effluent to the avoidance chamber. Using a recorder assembly developed by Scherer and Nowak, 1973, the total time spent in either pure water or effluent was measured.

Prior to each experiment fish were exposed to pure water for twenty minutes to habituate the fish to chamber conditions and to ascertain if there were any inherent preference or avoidance to an end of the chamber. No statistically significant bias was observed in the system. The introduction of water or effluent from each end was alternated to eliminate any possible bias.

Four concentrations of effluent were tested at 15°C to determine avoidance: preference response. For mine A the concentrations were 1.0%, 10.0%, 30.0%, and 50.0% of the effluent. For mines B, C, and D the concentrations tested were 1.0%, 10.0%, 50.0% and 100% of the effluent.

Chemical analysis of the effluent was performed by the Environmental Protection Service, Winnipeg. The pH, turbidity, and conductivity were measured for each test concentration.

A total of ten fish per concentration were tested, except for the 100% concentration of mine B where only five fish were tested due to a lack of effluent. Each fish was tested only once and was then discarded.

An analysis of variance was used for each effluent series to determine whether there was statistically significant avoidance or preference, using % time spent in pure water, relative to the control group. The no-effect (threshold) concentration was then determined for the effluent series. Finally, mean percent time spent in pure water ± one standard error was plotted against log concentration effluent.

RESULTS

The results are itemized in Table I. The 96 hr LC_{50} for mines A, C and D were >100%. The 96 hr LC_{50} for mine B was between 85.0 and 100%.

The mean % time spent in pure water decreased as the concentration of effluent increased for mines A, C, and D (Fig. I, II, III resp.). The effluent concentrations elicited significant ($P < .001$) preference responses compared to the respective control values. Significant ($P < .05$) threshold preference responses were determined for the three mines. For mines A and C the threshold response occurred between 1.0% and 10.0%, while for mine D it occurred between 0% and 10.0%.

For mine B the effluent concentrations elicited a significant ($P < .001$) avoidance response compared to the control values. This avoidance was not correlated with increasing effluent concentration (Fig. IV). A significant ($p < .001$) threshold response occurred between 0% and 1.0%.

Chemical tests were performed for pH, conductivity, and turbidity (Table 2) on the effluent concentrations. The pH level remained relatively stable for all mines. Conductivity increased as concentration increased for mine A, and to a lesser extent for mines B, C, and D.

DISCUSSION

The results indicate that effluents from three of the four metal mines significantly attracted whitefish. The preference response increased with increasing effluent concentrations. This preference,

SUMMARY FORM

	Concentration of Effluent (%)	# of Test Organisms	Mean % Time in Pure Water ± Standard Error
Mine Effluent #A	1.0	10	51.67 ± 4.95
	10.0	10	31.17 ± 5.15
96 hr LC50 = >100%	30.0	10	17.50 ± 3.60
	50.0	10	11.50 ± 3.28
Mine Effluent #B	1.0	10	58.00 ± 3.17
	10.0	10	57.67 ± 3.83
96 hr LC50 = 85-100%	50.0	10	50.17 ± 3.33
	100.0	5	57.50 ± 10.33
Mine Effluent #C	1.0	10	53.00 ± 3.67
	10.0	10	37.00 ± 4.50
96 hr LC50 = >100%	50.0	10	33.50 ± 3.33
	100.0	10	26.00 ± 3.50
Mine Effluent #D	1.0	10	47.00 ± 7.50
	10.0	10	42.50 ± 4.17
96 hr LC50 = >100%	50.0	10	33.33 ± 2.33
	100.0	10	28.50 ± 3.17

Table 1. Acute bioassay and avoidance:preference test results for Mines A, B, C, and D.

	Effluent Concentration	PH	Conductivity µmho/cm	Turbidity jtµ
Mine A	1.0%	7.61	140	3.2
	10.0%	7.70	140	25.0
	30.0%	7.53	122	63.0
	50.0%	7.59	127	122.0
Mine B	1.0%	7.62	120	2.7
	10.0%	7.66	203	6.5
	50.0%	7.54	560	4.3
	100%	6.81	920	27.0
Mine C	1.0%	7.65	100	2.7
	10.0%	7.63	150	2.5
	50.0%	7.87	303	3.1
	100%	7.89	550	6.3
Mine D	1.0%	7.65	111	4.0
	10.0%	7.74	140	4.5
	50.0%	7.64	270	6.3
	100%	7.85	430	6.3

Table 2. Chemical parameters monitored during avoidance:preference tests.

Figure 1

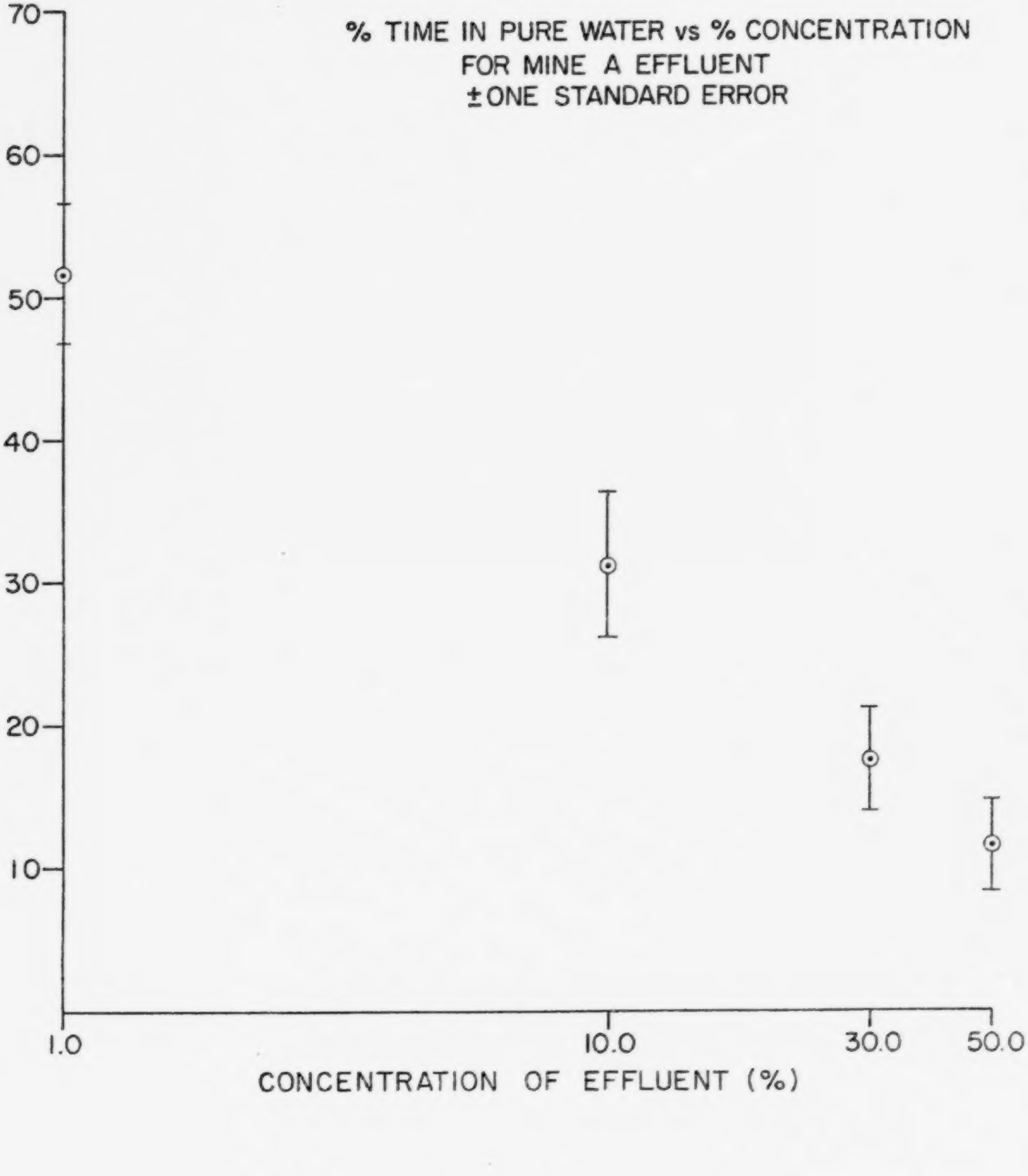

% TIME IN PURE WATER vs % CONCENTRATION
FOR MINE A EFFLUENT
±ONE STANDARD ERROR
70
60
50
40
30
20
10
1.0
10.0
30.0
50.0
CONCENTRATION OF EFFLUENT (%)

Figure 11

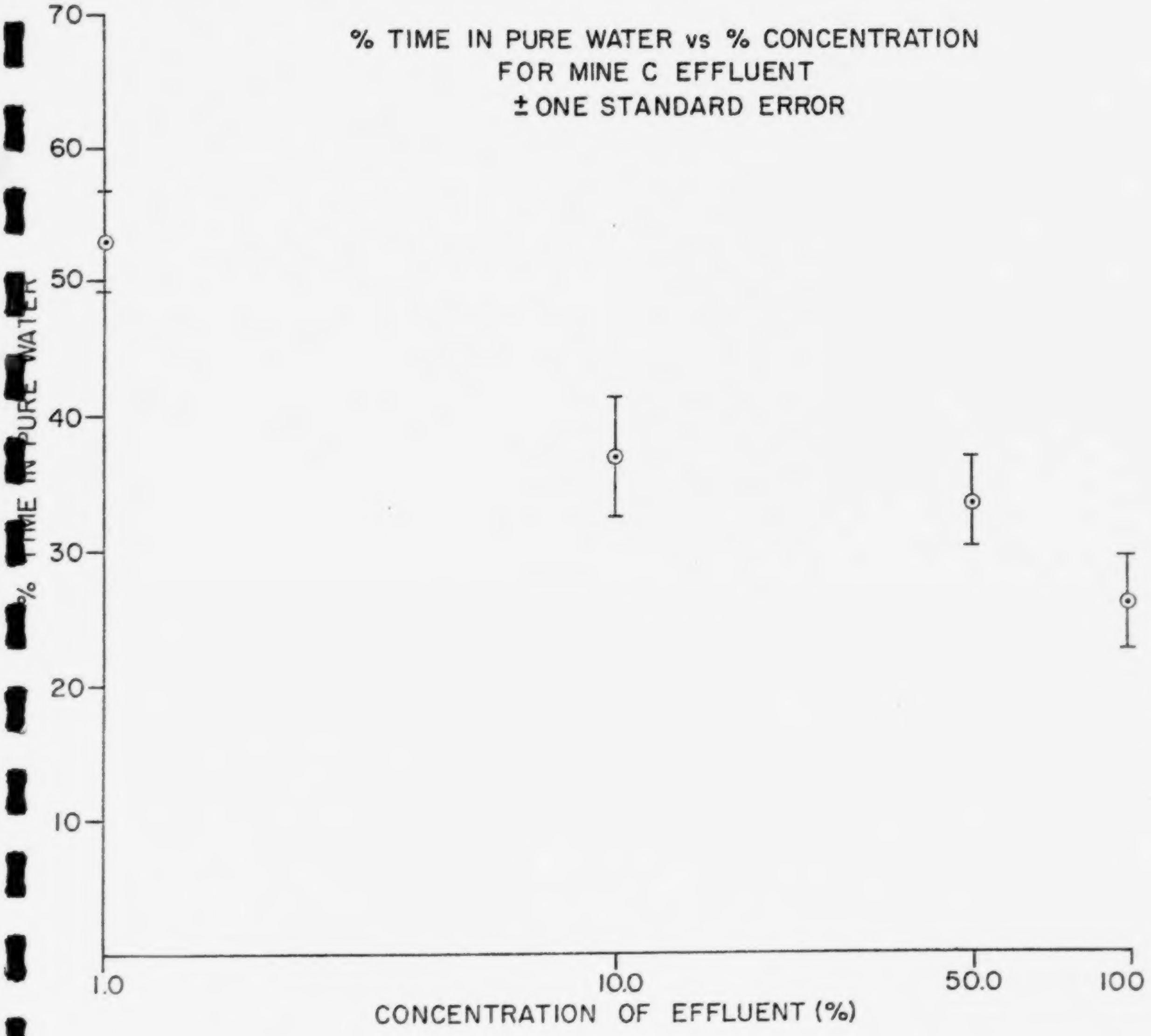

% TIME IN PURE WATER vs % CONCENTRATION
FOR MINE C EFFLUENT
± ONE STANDARD ERROR
% TIME IN PURE WATER
70
60
50
40
30
20
10
1.0
10.0
50.0
100
CONCENTRATION OF EFFLUENT (%)

Figure 111

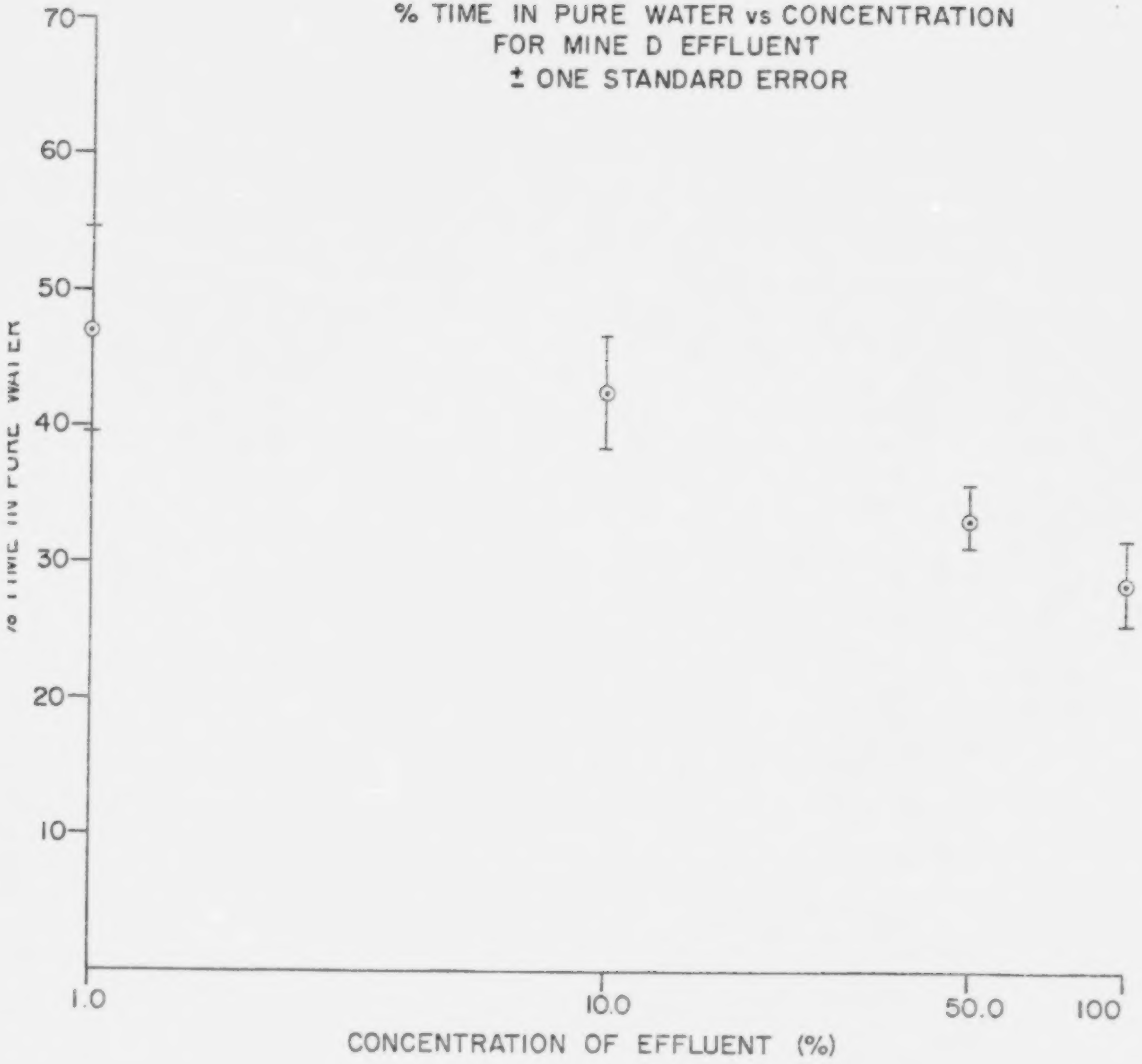
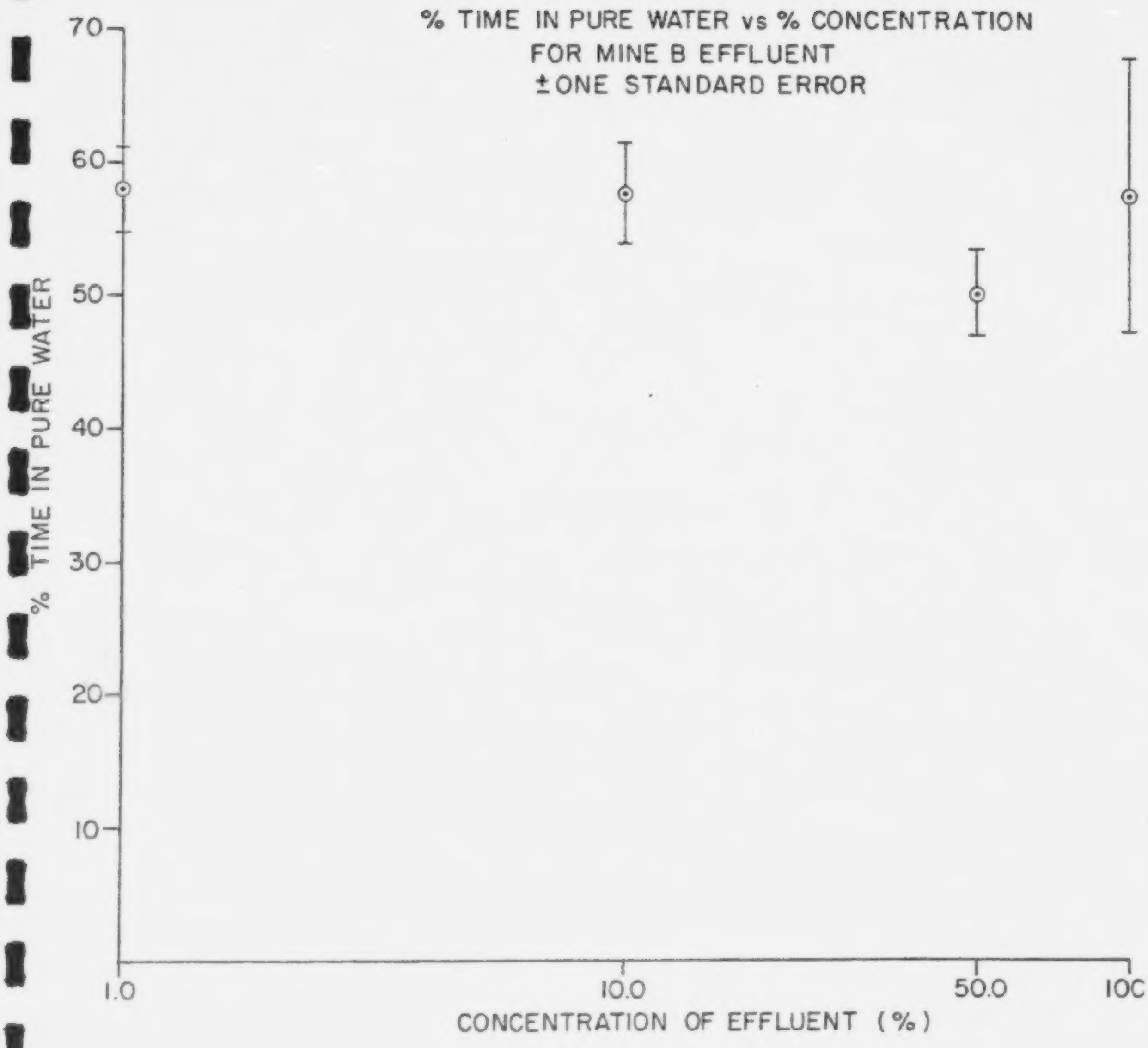

% TIME IN PURE WATER vs CONCENTRATION
FOR MINE D EFFLUENT
± ONE STANDARD ERROR
% TIME IN PURE WATER
70
60
50
40
30
20
10
1.0
10.0
50.0
100
CONCENTRATION OF EFFLUENT (%)

Figure IV


% TIME IN PURE WATER vs % CONCENTRATION
FOR MINE B EFFLUENT
±ONE STANDARD ERROR
% TIME IN PURE WATER
70
60
50
40
30
20
10
1.0
10.0
50.0
10C
CONCENTRATION OF EFFLUENT (%)

resulting in prolonged exposure to high concentrations of mine effluent, could in time cause physiological impairment or death to the fish. Clarke (1974), in a review, lists various sublethal effects associated with exposure to metal mine effluent components.

Mine B effluent elicited a significant avoidance response. The differences in response between mine B and mines A, C, and D could not be explained on the basis of the available chemical parameters.

Very little work has been done investigating the preference: avoidance response of fish to metal mine effluents. A study by Sprague and Saunders (1963) indicated that Atlantic salmon (Salmo salar) avoided base metal mine effluents. The difference in response to metal mine effluents between this study and our own may be due to the different test species or to differences in the chemical characteristics of the effluents.

The available literature is largely concerned with components of metal mine effluents. Sprague and Saunders (1963) reported that young Atlantic salmon detected copper and zinc in solution at concentrations of less than 10% of the incipient lethal level, and avoided the copper and zinc solution at 20% of the incipient lethal level.

Sprague (1968) reported that the threshold avoidance level for rainbow trout (Salmo gairdneri) to be 5.6μ gm/ℓ of zinc. Lawrence and Scherer (1974) reported that rainbow trout and whitefish responded similarily to drilling fluids. Hence the literature suggests that whitefish would avoid base metal mine effluents, but our results do not support this expectation for the majority of cases as there was a strong preference response to three of the four mines, whereas in the fourth there was a significant avoidance response.

Turbidity produced preference responses in some previous investigations, depending upon the species being examined and its habitat. (E.I.F.A.C., 1965. Lawrence and Scherer, 1974). Thus, turbidity may also be a significant factor.

Further studies to elucidate the role of turbidity in eliciting preference responses to whitefish are planned. Also, it might be useful to study artificial effluents, effluents containing several metals in various concentrations, to see if any pattern of avoidance or preference emerges from exposure to the ratios of metals.

Populations of whitefish may be attracted to potentially deleterious levels of metal mine effluent. Since whitefish are an important commercial fish in Manitoba and elsewhere, this could have serious economic consequences.

This study also emphasizes that screening an effluent with a routine 96 hr. lethal bioassay is not sufficient since three of the effluents studied were non-toxic, but still elicited measureable preference responses which could result in sublethal or chronically lethal effects to the fish.

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DISCUSSION OF SUB-LETHAL BIOASSAY

John Davis

Peter Hodson, I would like you to explain the rationale for your choice of enzymes. Would perhaps the measure of simple hemoglobin concentrations suffice to demonstrate the sub-lethal effect in this case.

Peter Hodson

Part of the problem with enzymatic studies is that inhibition of enzymes is not necessarily an indication of an effect. The reason we chose this enzyme was that it has been shown in mammalian systems to be extremely sensitive to lead and it is used in screening programs for detecting lead exposure in children and occupationally exposed workers. What they have also found is that in the synthesis of heme the enzyme of interest (δ - aminolevulinic acid synthetase) is the rate limiting enzyme for the production of heme. We seem to be able to get massive inhibitions of δ - aminolevulinic acid dehydrase without any real change in hematocrit haemoglobin concentrations. The latter enzyme is such an historically old enzyme in terms of biological development that it has built into it a massive reserve activity such that it can be inhibited without seriously reducing the production of heme. We have been looking at hemoglobin haemotocrit red blood cell counts and there is no real evident effect after 16 weeks exposure to lead.

Ray Brouzes

Peter Hodson, a bioassay to my mind is a total animal response. If we measure one parameter with that animal we may be missing a response in some other system or some other enzyme. I am not clear on the distinction between using a biochemical reaction compared to an entire animal response.

Peter Hodson

I think that Mount and Stephan in 1967 said that even a good physiologist would be hard pressed to state what importance a 10% change in hematocrit would be to a fish. That is why I feel that a short-term physiological response should be compared to a life cycle study. I am not saying that inhibition of the enzyme is the animal's toxic response, I am saying that it seems to be very closely correlated to the animals ultimate response. I think you have to be very careful to outline several criteria for these shortcut methods.

Gordon Craig

I support this approach because we have seen members of our own group measure sublethal responses and automatically assess them as deleterious. I think that sublethal data should be presented in association with mortality or reproduction data such that the relationship between the responses can be established as a constant. Only then can that sublethal response be considered deleterious.

Henry Majewski

We would use avoidance studies to establish testing priorities. If an organism enters into an effluent instead of escaping from it, chronic exposures of fish to the effluent should be carried out.

Don McLeay

Peter, was there any change in enzyme activity after less than one week of lead exposure? Did you relate your response to fractions of a 96-hour LC-50?

Peter Hodson

We have not looked at a shorter exposure period than seven days. There are reports in literature on mammals that inhibition of this enzyme in human blood will occur within a day or two of exposure. Inhibition of the enzyme in our fish occurred between 1/100 and 1/1000 of the 96-hour LC-50 concentration. The 96-hour LC-50 for lead is an academic number because water chemistry controls the solubility and consequently the availability of lead to the organism.

Ed Pessah

Mr. Majewski, I have noticed that in similar physiological studies there is an initial increased response then a reduction of that response. You seem to show that in your data as well. Can you make any mileage of that sort of information and relate it to something that might happen at a later point in time.

Henry Majewski

I can't make much out of it other than the fish probably acclimate over a 24-hour period.

John Toby

Mr. Supeene, was there any difference in the water chemistry between the holding and testing conditions.

Ken Supeene

The same water was used in both cases.

Gerard Leduc

Mr. Supeene, how is the fish introduced into the chamber?

Ken Supeene

The fish is placed in the chamber and clean water flows through both sides for twenty minutes. During the last ten minutes the fish's movements are plotted to determine whether there is a bias for one side of the chamber or the other. The effluent is then introduced through one end of the chamber.

Ed Pessah

Mr. Supeene, last year there was an article published on drilling mud effluents and the authors referred to preference tending towards avoidance responses in fish. They suggested that the fish were more comfortable in the more turbid situation. Has this happened in your case? Has this been checked using something that would cause turbidity but no chemical threat?

Ken Supeene

In previous reports I have read turbidity does cause preference responses and we have noticed preference tending to avoidance with higher concentrations. We saw only preference responses in these studies.

Otto Langer

Mr. Supeene, what attempts have been made or are any attempts being made to relate your laboratory results to the actual field situation?

Ken Supeene

This is part of a larger laboratory study of various species responses, behavioural studies and invertebrate bioassays. A few toxicity bioassays and stress bioassays have been carried out in the field. We shall try to correlate all of this information in our final report.

Gerard LeDuc

We tend to focus our attention on short-cuts and try to get the answers to long-term effects but we should maintain and intensify the long-term studies which will provide the ultimate answers. We have to impress upon the decision making bodies that a comprehensive understanding of growth, reproduction, swimming ability, respiration and behaviour is more important than a short-term response. Short term response would be more meaningful if carried out in parallel with long-term studies. We also have to carry out the same sort of studies with other freshwater and marine aquatic organisms besides fish.

TOPIC IV

FIELD BIOASSAY

A MOBILE BIOASSAY LABORATORY

by

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A MOBILE BIOASSAY LABORATORY

INTRODUCTION

Effluent regulations and water quality objectives have been based on laboratory toxicity results. Therefore it is most important to determine if there is any relationship between toxicity measured in the laboratory and toxicity measured in the receiving water to ensure that effluent regulations and water quality objectives will protect aquatic biota.

The conventional method of determining the toxicity of an industrial effluent is by either static or continuous flow bioassays in a central laboratory. The problems with this are:

1) Diluent water quality in the laboratory may differ from that in the receiving water.
2) Large volumes of effluent may have to be transported to the laboratory, usually at great cost.
3) Effluent toxicity may change during storage in the laboratory.
4) Changes in effluent toxicity due to process changes may not be detected by a single sample.

Since wastes discharged from metal mines are one of the major sources of pollution in Canada, especially in the Central region, mine effluents were chosen for the laboratory-field comparison.

In this paper, I will discuss the requirements for the mobile laboratory unit, type of bioassays performed, the design of the mobile laboratory unit, problems encountered, and recommendations on the practicality of using a mobile bioassay laboratory.

REQUIREMENTS FOR THE MOBILE BIOASSAY LABORATORY:

The mobile bioassay laboratory was designed to meet the following requirements:

1) The bioassay laboratory must be able to operate with minimum external services.
2) It must be able to be re-located within 2-3 days.
3) It must be able to survive extreme road conditions while travelling to and from a site.
4) It must be large enough to transport in excess of 6000 litres (1200 gallons) of effluent to the Freshwater Institute.
5) It must be versatile to allow changes in the type and number of bioassays performed on site.

With the above requirements in mind, several alternatives were considered, including a 52 foot mobile field laboratory trailer used by Lake and Loch (1973), a camper unit on the back of a truck, and a five-ton truck with a 20-foot enclosed cargo area. We decided to use a five-ton truck with the 20-foot long enclosed cargo area mainly because of its mobility and cargo capacity.

TYPES OF BIOASSAYS

Four types of bioassays using two different test organisms were performed:

1) Continuous flow temperature controlled bioassays, using rainbow trout (*Salmo gairdneri*) and freshwater shrimp (*Gammarus lacustris*).
2) Activity stress continuous flow bioassays at ambient temperature using rainbow trout.

3) Static bioassays with an artificial effluent at ambient temperature using rainbow trout.

4) Oxygen limiting bioassays at ambient temperature using rainbow trout.

In the continuous flow bioassays, both rainbow trout and freshwater shrimp were placed in the same test tank. The freshwater shrimp were separated from the rainbow trout by the placement of freshwater shrimp inside a cage, made of small mesh, screen which was then placed in the larger bioassay vessels containing the rainbow trout. A modified proportional dilutor (Mount and Brungs 1967), containing a stand-pipe siphon cap which could be replaced to produce different effluent concentrations was used to ensure a continuous delivery of toxicant and dilution water to each test vessel.

Activity stress continuous flow bioassays which used the overflow from the continuous flow bioassays, were performed in 40 litre circular swimming troughs. In the trough, the fish were forced to swim against a constant current of approximately 32 cm/sec. This test measured the toxicity of the effluent with an added physical stress on the fish.

A static bioassay was performed to determine the influence of changes in receiving water chemistry on the toxicity of a make-up mine effluent containing copper, nickel and zinc to rainbow trout.

Oxygen limiting bioassay measured the oxygen consumption rate per gram of fish (Carter 1962). An erlenmeyer flask with a rubber stopper was used to perform the test. Initial and final dissolved oxygen were measured and recorded and the time required for each fish to die was noted.

FLOOR PLAN OF LABORATORY

With less than 14 sq. m (160 sq. ft) of area available for the laboratory and effluent holding facility, we had to maximize the usage of the available space. A 3000 litre fibreglass tank was used to store effluent, it was located at one end of the laboratory. The continuous flow bioassay bath, work bench, and one set of the circular swimming troughs were located at the other end of the laboratory. The static bioassay containers and the second set of circular swimming troughs were located near the middle of the laboratory (Figure 1). A 25,000 BTU/hr portable air conditioner was installed at one end of the laboratory. The interior of the laboratory was insulated with polyurethane.

DILUENT WATER, EFFLUENT, AND ELECTRICAL SUPPLY

Water was either pumped directly to the laboratory from upstream of the waste discharge, or was pumped into barrels from the receiving water and then transported daily to the laboratory. Effluent was supplied to the laboratory in a similar method. Major problems may occur if it is necessary to obtain effluents from a submerged or enclosed discharge or if no access is available to the point of discharge.

The mobile bioassay laboratory required an electrical system of 220 volts, single phase, A.C. at 40 amp. The power can be provided in one of three methods:

1) A direct in-plant connection to the commercial power supply. This is the most desirable method.
2) The second alternative is to use a 10 KW gasoline or diesel powered A.C. generator. This method is the least desirable of the three methods because of the problems encountered with

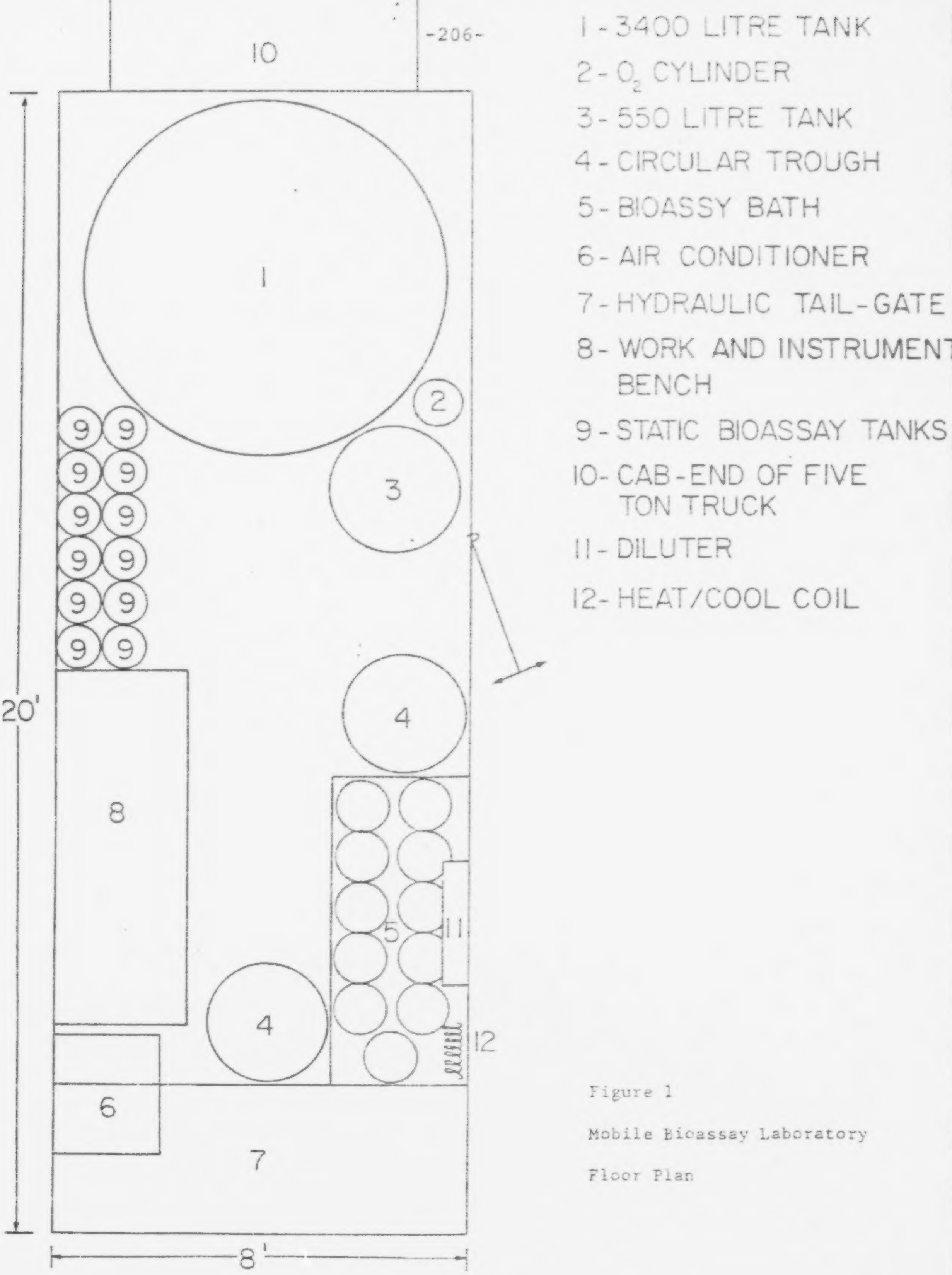
10
1
2
3
9
4
8
5
11
12
4
6
7
20'
8'
1-3400 LITRE TANK
2-O_2 CYLINDER
3-550 LITRE TANK
4-CIRCULAR TROUGH
5-BIOASSY BATH
6-AIR CONDITIONER
7-HYDRAULIC TAIL-GATE
8-WORK AND INSTRUMENT BENCH
9-STATIC BIOASSAY TANKS
10-CAB-END OF FIVE TON TRUCK
11-DILUTER
12-HEAT/COOL COIL

Figure 1

Mobile Bioassay Laboratory

Floor Plan

transportation, re-fuelling, maintenance, noise level and exhaust fumes.

3) The third method is a field hook-up to the high voltage power line. This is the most expensive of the three methods.

For our short term study outlined here, only methods (1) and (2) were employed because of the high cost of method (3).

DISCUSSION

Last summer's study on the mine effluents using the mobile bioassay laboratory indicated that it is feasible to conduct bioassays during a short term field study. Each mine was studied in less than two weeks, including the travelling time, time required to set up the laboratory on site, conduct the tests and disassemble the laboratory. The cost of the study, involving four sites was $17,000 (Table 1) of which $11,400 was non-recoverable (rental of equipment, accommodation expenses, etc.)

A minimum of two persons were required to operate the mobile laboratory in the field. A third person would have been useful to assist in the driving, in setting up the bioassays and in effluent collection. The third person could be part of a benthic or fish sampling contingent working in conjunction with the bioassay investigation.

One of the problems encountered during the study was the mortality of rainbow trout during transport and acclimation. Fish were transported to the site by scheduled airline or via the mobile bioassay laboratory in plastic bags inflated with oxygen and sealed in coolers at 1°-5°C. Many of the fish transported in this manner died, apparently due to a change in the physical properties of the receiving water or insufficient

TABLE 1

Budget

Item	Operation & Maintenance	Capital
Lease - 5 ton truck	$ 4,000	
Lease - 3/4 ton truck	1,200	
Rental - generator	1,200	
Bioassay bath		$ 500
Pumps and tank		2,000
pH meter		700
Oxygen meter		500
Weight balance		500
Air conditioner		500
Heat/cool unit		800
Air compressor		200
Field accommodation and expenses	4,000	
Miscellaneous	1,000	
	$ 11,400	$ 5,700

acclimation to temperature changes. The latter problem occurred because of the limited time available for acclimation (2-3 days) prior to the beginning of a bioassay. Excessive mortality didn't occur during the transport or acclimation of the invertebrates.

To avoid any abrupt temperature change during acclimation, it would be advisable to begin temperature acclimation during transport. This could be accomplished in a temperature controlled cargo area which is optional on many large truck rentals. Temperature control would also permit cooling of the effluent being returned to the laboratory to reduce effluent degradation.

There are several improvements which could be incorporated into the mobile laboratory. These are:

1) Increasing the size of the laboratory or removing the 3400 litre effluent holding tank from the laboratory and using instead a tank trailer used exclusively for effluent transport. This would allow more bioassay, storage and desk space.
2) On warm days >25°C, the cooling capacity of the air conditioner was exceeded and the temperature in the bioassay vessels rose in response to the increase in air temperature. Increasing the amount of insulation and providing an air lock area such as a porch would ameliorate temperature fluctuations.
3) The modified proportional dilutor used in this study required constant recalibration and repairs due to exposure to rough road condition. Using plexiglass material to build the dilutor would reduce this problem, or a sturdier diluter such as that described by R. Brouzes, R. Neufeld, H. Altosaar at the Domtar

Research Center, Montreal (Personnel Communication 1975). This diluter might be more applicable for field bioassay investigation.

For longer term studies, a larger mobile laboratory such as described by Lake and Loch (1973) would be more desirable and would allow better control of environmental variables. However, for studies of less than one month, especially in remote areas, the mobile laboratory described in this paper is more suitable.

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GROWTH EXPERIMENTS AS A FIELD STUDY

by

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GROWTH EXPERIMENTS AS A FIELD STUDY

INTRODUCTION

Over the years considerable data have been gathered on the acute toxicity of many compounds and effluents. Much less information is provided in the literature concerning subacute or sublethal responses. Some of the sublethal responses that have been monitored are swimming performance, avoidance reactions, respiratory changes, reproductive impairment, alteration of blood chemistry and flavour impairment of fish flesh (Howard et al, 1972; Sprague and Drury, 1969; Walden et al, 1970; McKim and Benoit, 1971; McLeay, 1973; Cook et al, 1971).

Growth rate, in conjunction with food conversion efficiency is an additional sublethal study that can be undertaken in a relatively simplistic manner. Many investigators have developed growth studies into complex bioenergetic evaluations. This type of study is often not practical for field applications where one wishes to monitor the sublethal effects of an industrial effluent.

What I wish to discuss in this paper are the basic principals involved in conducting a growth experiment in the field and relate them to field studies performed by this laboratory. Two significant variables which will be discussed are temperature and feeding rate. I would also like to pose some questions arising from this methodology.

Materials and Methods

Table 1 presents some of the basic steps that should be included in a standardized growth experiment. The use of an antibiotic treatment is often a contentious point. It is impossible to derive reliable data from a sublethal bioassay when your test species is unhealthy and stressed. Therefore, it is recommended that a prophalactic treatment be administered two weeks prior to the initiation of the test.

The size of the sample to be tested must be large enough to provide sufficient specimens for meaningful statistical interpretation. We have found that twenty fish per concentration with the concentration tested in duplicate is a workable number.

TABLE 1 MAJOR ASPECTS OF A GROWTH EXPERIMENT

1. ANTIBIOTIC TREATMENT
2. SELECTION OF SAMPLE
3. PRE-EXPOSURE MEASUREMENTS
4. PLACEMENT OF EXPOSURE TANKS
5. ALLOTMENT OF FISH
6. PRE-OBSERVATION EXPOSURE PERIOD
7. DURATION OF EXPOSURE
8. FEEDING RATE
9. PHYSICAL AND CHEMICAL CONDITIONS OF EXPOSURE:
   DISSOLVED OXYGEN, TEMPERATURE, LIGHTING
   CONDUCTIVITY, pH, TANK LOCATION

Pre-exposure measurements of length and weight must be made on each individual fish in order to determine that the population of fish chosen are similar in size at the beginning of the exposure period. The position of the tanks within the experimental bay and allotment of the fish among these tanks must be done in a random manner.

It is recommended that there be a 24-hour pre-exposure observation period prior to introduction of the toxicant into the system. Any random mortality resulting from excessive handling during measurement phase will be observed in the first 24-hours. Other aspects of a growth study that should be standardized are duration of exposure and feeding rate expressed as a percentage of body weight per day.

Physical and chemical conditions of the exposure that must be monitored are dissolved oxygen, temperature, conductivity, pH and light intensity. All tanks within the experimental bay must be shielded from extraneous sources of lignt and mechanical disturbance.

Natural or man-made disasters are a consequence of conducting continuous-flow experiments in the field. Obviously, adequate planning may eliminate most of these problems. A concentration range for growth studies on industrial effluents may include a maximum concentration in which periodic partial mortality occurs. When partial mortality occurs a decision must be made whether or not to replace these dead fish. If the fish are not replaced a proportional reduction in the tank volume and feeding rate must be made. If the fish are replaced the new fish must be individually identified. Several methods for identification of individual fish have been discussed in the literature. Some of these methods include the use of fluorescent pigments, cold branding, tattooing and jaw tagging (Refstie and Aulstad, 1975). The use of jaw tags is unadvisable in growth studies as it may restrict feeding activity. After any fish is branded there should be a 24-hour delay before the fish is placed in the growth aquaria.

If the total mortality occurs in any concentration, the fish should be replaced and the frequency of mortality recorded. In this way a single sublethal experiment may provide both an LC_{50} figure and an effective sublethal concentration.

In the event of a complete power failure the dosing system must be designed to stop the introduction of the toxicant and dilution water into the system. The dissolved oxygen concentration of the exposure tanks can then be maintained by means of compressed air cylinders and solenoid valves.

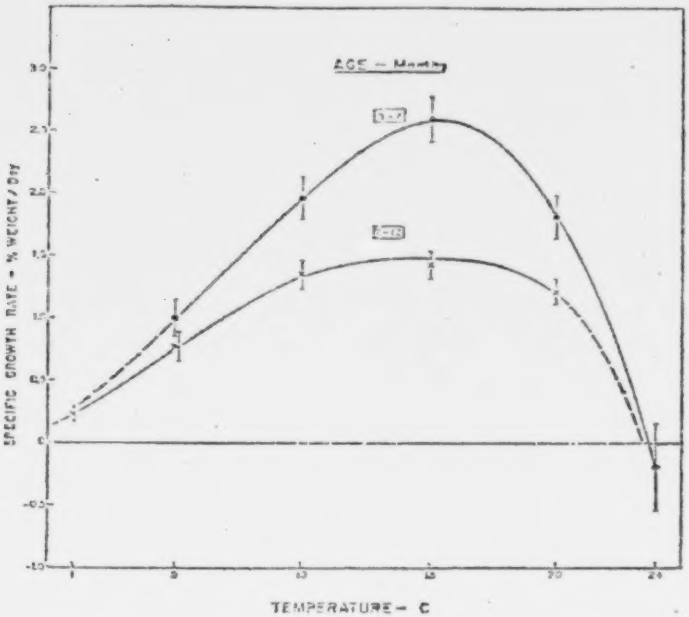
Field growth studies were conducted by this laboratory at a pulp and paper mill on Lake Superior in Northern Ontario. The facilities consisted of a small mobile laboratory primarily used for simple routine chemical analysis. The bioassay unit was situated in an adjacent pre-fabricated garage. This bioassay building took approximately three weeks to construct including installation of plumbing and electrical services. With the short field season experienced in northern areas the advantages of having a single mobile bioassay module with suitable environmental control are clear.

The receiving water for the effluent discharge was utilized as a source of dilution water. Temperature regulation of the dilution water was accomplished by means of two 1 h.p. refrigeration units in conjunction with two 1000 watt electric immersion heaters. This unit supplied water for maintaining fish stocks and dilution water for continuous flow bioassays. Since the effluent being studied was a heated discharge the possibility of a temperature gradient across the concentration range existed. Therefore, the effluent was continuously pumped through 60 feet of small bore stainless steel tubing immersed in the temperature regulation bath, prior to its discharge into the dilution system.

Figure 1, from Brett, Shelbourn and Shoop (1969) demonstrates the dramatic effect of temperature on growth rate. In order to ensure that an observed change in growth rate is a result of the sublethal effects of an effluent and not temperature fluctuations, the temperature regulation system must be of sufficient capacity to negate the effect of seasonal and diurnal water temperature variations.

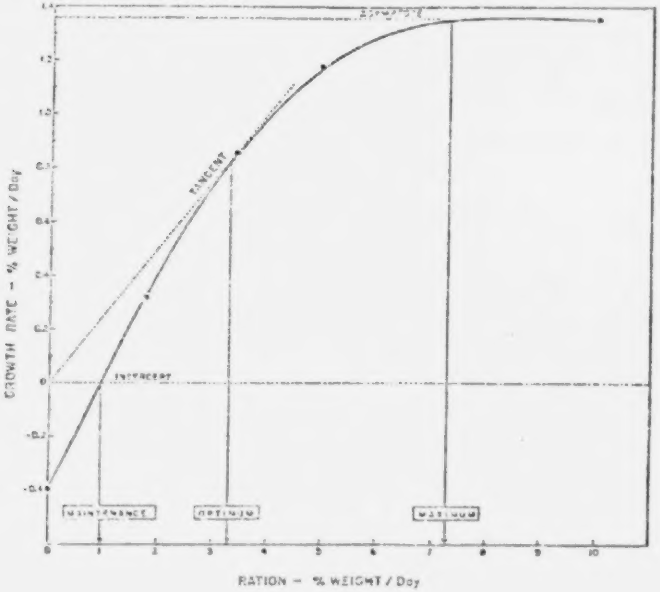
The effluent dosing system consisted of a large automatic pipetter. Both the rate and volume of delivery of the pipetter could be varied to produce the range of effluent concentrations desired. Dilution was accomplished by means of a modified Mount and Brungs diluter with a flow-rate of approximately 450 mls. per minute. Lighting was provided by a combination of incandescent and flourescent lights. A light controller,

Fig. 1: Relation between temperature and growth rate (+2 S.E.) of young sockeye salmon fed on excess ration.



(From Brett, Shelbourne and Shoope, 1969)

Fig 2: Effect of ration size on growth rate of young sockeye salmon.



(From Brett, Shelbourne and Shoop, 1969).

modified after Drummond and Dawson (1970) maintained a 16-hour photoperiod with a dawn and dusk simulation of light intensity. Dissolved oxygen concentrations in the exposed tanks were maintained in excess of 90% air saturation by means of a compressor and a single airstone in each aquaria.

Food types and feeding rates are the most important variables in a growth experiment. Figure 2 from Brett, Shelbourn and Shoop (1969) demonstrates the effect of different feeding regimes. A feeding rate of greater than 7% of body weight per day produced the maximum growth rate. The gross food conversion efficiency of an organism is the change in the observed weight of the organism divided by the total weight of the food eaten. Determining the exact weight of food eaten by the fish is often impossible because some of the food placed in the tanks may settle out before it is eaten. A partial solution to this problem is to increase the daily frequency of feeding with a proportional decrease in the amount of food per feeding.

DISCUSSION

Is a growth experiment a viable field study? Certainly it is not a tool to be used to monitor a waste where there are extreme fluctuations in toxicity. Considerable time must be devoted to setting up a growth experiment in the field with a great deal of emphasis being placed on designing an adequate water supply and a reliable temperature regulation system.

The advantages of undertaking growth experiments in the field are significant. The possibility of detoxification of the waste during transport back to a central testing facility is eliminated. Also, by continually sampling the effluent on-site all fluctuations in waste quality are immediately monitored. On-site testing takes into account the quality of the receiving water.

Isa growth experiment a feasible approach for a regulatory agency? Present legislation on the Federal level sets limits only for acute toxicity. Is acute toxicity the only criteria for monitoring waste discharges or do we have a responsibility for detecting any impairment in water quality and thus determine zones of influence? What is a significant level of growth impairment and over what time period should it be monitored? Conversely is growth impairment a reliable indicator of impaired water quality? Are pretreatment methods such as neutralization, aeration or filtration to be applied to the waste before it is tested?

Investigators must evaluate each waste and its respective receiving water separately and then assess which of the preceeding questions is relevant to that particular situation. Some standards must also be set on the nutritional value of foods used in growth experiments and the ration level used.

CONCLUSION

In summary growth experiments conducted in the field may be a valuable tool as long as the inherent limitations are realized and the guidelines proposed are strictly adhered to. In order to apply the results from growth appropriate legislation must be developed with respect to effluent discharges.

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THE FLAGFISH (JORDANELLA FLORIDAE)
USED TO ASSESS THE EFFECTS OF CONTAMINANTS
ON REPRODUCTION UNDER FIELD CONDITIONS

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INTRODUCTION

The disappearance of fisheries and reproduction impairment of fish associated with low pH waters have been documented by many authors, Mount (1973), Johnsson (et al 1973), Beamish (1974). The study was initiated to determine whether pH effects were augmented under soft water conditions commonly found in Northern Ontario and it was hoped that the life cycle stages of fish development sensitive to depressed pH could be identified.

The intent of this paper is to identify the reproductive responses of flagfish (Jordanella floridae) that can be monitored and to provide some indication of relative degree of their sensitivity.

METHODS

A mobile laboratory was established at L. Panache (81° 20.6' N longitude, 46° 16.4' E. latitude) which is a precambrian lake with a quartzite and limestone basin. Lake Panache has a hardness of 28 ppm as $CaCO_3$, alkalinity of 11 ppm as $CaCO_3$ and supports a bass fishery with some lake trout and pickerel.

Water from the lake was pumped to a head tank supported beside and above the lake on a metal stand. The incoming water was maintained at a temperature of 27°C by electrically heating the head tank system with a 3000 watt chromalux-immersion heater, regulated by a Chromalux-Fenwal temperature controller. Further temperature control was implemented by a 100 watt immersion heater in each exposure tank. No cooling system was required as the incoming water never exceeded 27°C. The pH of the incoming lake water (pH 6.8) was adjusted by the addition of 0.1N sulfuric acid stock, injected by an Oxford automatic pippetor. Acid injection, signaled by a delayed relay switch, occurred just prior to the introduction of water to the mixing cell. The dilutor discharged one litre of water per channel every 2 minutes, and a flow splitter fed 2 duplicate 40 litre exposure tanks at each nominal pH. A Leeds and Northrop pH controller-monitor continuously recorded the pH in one of the lowest pH exposure tanks and provided a fail-safe function, by maintaining the pH between 4.4 and 4.6 in those tanks.

The remaining pH levels were monitored by a Radiometer M61 pH meter with a Bach-Simpson SAS 1 six channel switcher. The pH signal was recorded on one channel of a two channel Linear Instruments Corp. model 222 linear recorder. The second channel of the recorder was connected to the YSI model 47 eleven channel telethermometer thereby giving a continuous record of the temperature in each of the tanks. When the pH control system was used in a similar experiment incorporating Toronto municipal water (hardness 135 ppm as $CaCO_3$) pH control was more difficult to maintain especially around pH 5.

The bioassay species of choice was the flagfish (Jordanella floridae) as its short life cycle rendered it ideal for reproductive studies in the field. The flagfish used for bioassay were cultured according to Smith (1973). Fish were allowed to acclimate to the control water for a minimum of 14 days prior to use. They were maintained at a temperature of 27° and fed ad libitum 4 times daily, on a diet consisting of chopped rinsed earthworms and frozen brine shrimp (Artemia sp). Males and females were held in separate tanks to induce more rapid spawning upon initiation of the bioassay. Two males and six females were randomly selected and placed in each exposure tank. Control lake water flowed through the tanks for 5 days to establish that all exposure groups were capable of spawning and producing viable eggs. During this period any mortalities were replaced. Once the pH adjustment was initiated, mortalities were not replaced. The pH was then linearly depressed over a period of 5 days to the prescribed levels of pH 6.0, 5.5, 5.0, 4.5. The control tanks were supplied with lake water only. Acid exposure continued for 21 days. The tanks were cleaned daily and the pH, D.O. and temperature were recorded.

Eggs were collected, counted and inspected under a stereo microscope for fertility. Fertile eggs were those considered undergoing active embryological development, devoid of abnormalities such as reduced yolk size, lack of blastodisc, or opaque materials in association with the blastodisc or embryo. A maximum number of 50 fertile eggs collected were placed in floating nylon mesh baskets, and returned to their respective tanks to hatch. Eggs were incubated in the baskets for 5 days after which they were removed and the fry counted.

The ovaries and testes of the mated flagfish were then removed, fixed in Bouin's and cleared in 70% ethanol. The tissues were sectioned, stained in Harris's Haematoxylin and counter stained in Bowie's Eosin.

The growth study of hatched fry was initiated by randomly selecting 50 fry from each treatment and returning 25 fry to each respective treatment tank for rearing. Growth exposure continued for 6 weeks. Live measurements of the fry were taken from weekly photographs of the fish in a porcelain bowl containing a measured rule and the appropriate exposure tank water.

RESULTS AND DISCUSSION

The number of eggs produced under different pH conditions (Fig. 1) were reduced as the pH decreased. This parameter was easily recorded and proved to be a fairly sensitive response since impairment was observed at pH 6.0 when compared to the control conditions of pH 6.8.

The eggs being only about 1 mm in diameter were easily inspected and the stage of embryonic development could be readily identified. Fertility of the eggs was also reduced as the pH declined (Table 1) and impairment was also observed under pH 6.0 conditions.

The hatching success of eggs incubated in floating baskets proved to be variable and consequently no differences among treatments could be identified. An attempt was made to follow the "Duluth" recommendations of dipping the baskets and eggs during incubation as opposed to letting them float but this provided no improvement in the response. The Duluth Water Quality Laboratory also routinely dips eggs in a malachite green solution prior to incubation and upon any indication of fungal infection. This treatment regime was not considered suitable for our purposes.

Fry survival at the lower pH levels of 5.5 and 5.0 was markedly reduced during the first two weeks while controls and pH 6.0 fry suffered only about 10% mortality (Fig. 2). This period of yolk sac retention and development just prior to the "swim-up" stage appeared to be most sensitive to depressed pH. After this two week period there was little change in fry mortality for the remainder of the exposure period.

Growth rates of the fry based on dorsal image area data were not affected in the pH 6.0 group compared to controls while growth rates under pH 5.5 and pH 5.0 conditions were reduced (Fig. 3). However, final weights of all treatment groups were significantly less than controls (Table 2).

FIG. 1 Daily Egg Production of Flagfish Communities Held Under Depressed pH Conditions

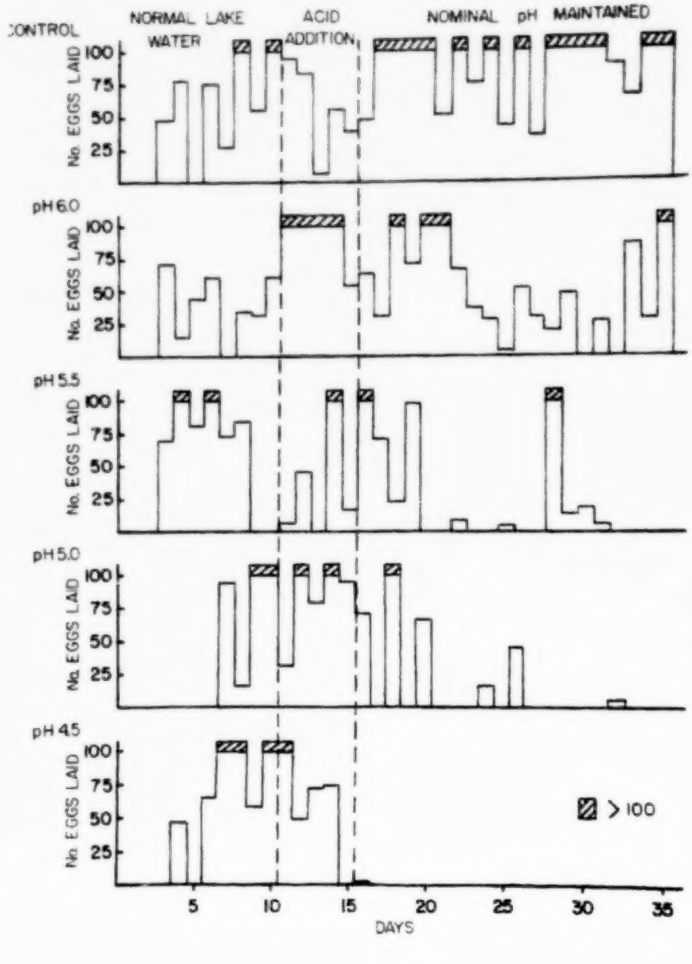
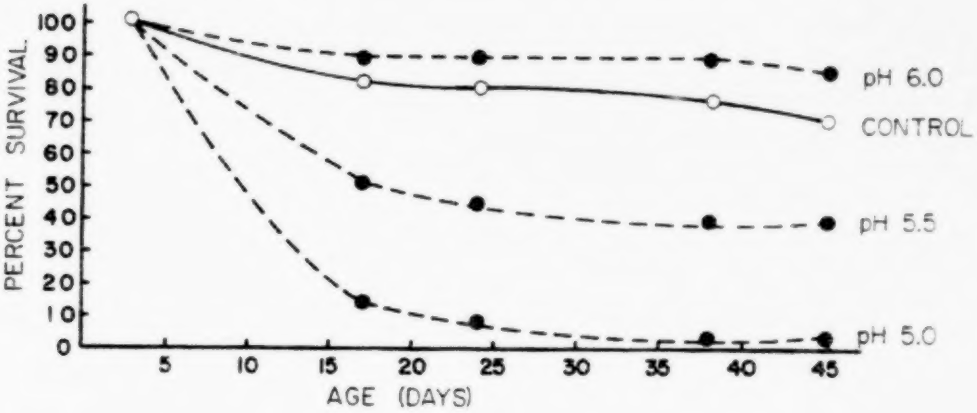


FIG. 2: SURVIVAL OF FLAGFISH FRY IN DEPRESSED pH CONDITIONS



TOPIC V

INVERTEBRATE BIOASSAY AND CULTURE

INVERTEBRATE BIOASSAY - WHY NOT!

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the environment. Invertebrate maintenance and toxicity testing may present problems when species are selected which have water chemistry and current requirements, food requirements, vegetation and substrate preference or intricate behaviour patterns (Oseid and Smith, 1974; Kanstantinov, 1972; Wallace et al., 1975). An increase in temperature from the field to laboratory may induce stonefly, damselfly or mayfly nymphs to emerge. Predacious invertebrates are sometimes difficult to hold when a live food source is not available. Invertebrates such as snails are tolerant to handling and sudden change in the environment.

(c) Ease of culture. Invertebrate species such as *Daphnia* may be reared in static conditions with little maintenance. *Gammarus lacustris* and *Hyalella azteca*, two of my major test organisms, may be maintained and reared in the laboratory. Other species such as mayflies, chironomids and crayfish have environmental requirements or behavioural patterns which result in unsatisfactory laboratory maintenance. At present there is a need for continued research into optimal culture conditions for reference invertebrates.

(d) Tolerance of the invertebrate to various toxicants. The known sensitivity of various invertebrates may be a factor when considering possible test organisms for a bioassay. In a study done by Rehwoldt et al. (1973) *Gammarus* and *Chironomus* were more sensitive to heavy metals than other invertebrates. This information may be considered in the selection of species sensitive to heavy metals in a mining study.

(e) Is the test invertebrate representative of the receiving water? During an impact assessment study if a particular invertebrate forms a significant proportion of the benthos to be receiving a contaminant it may be beneficial to use this species in effluent toxicity evaluation.

There are several problems associated with invertebrate toxicity testing. A major problem is control mortality. In industrial effluent study conducted by our group last year, control mortality occurred with greater frequency during invertebrate bioassays (mean invertebrate control mortality 5.84%, rainbow trout <1%). Ten percent has been cited as an acceptable control mortality figure for invertebrates as reported by Nebeker (personal communication with regard to amphipods) and Biukema et al. (1974). Control mortality is a problem because it is difficult to assess effluent toxicity when other factors appear responsible for mortality. These factors include change in environmental requirements, age of the test organism, reproductive status of females, health of a lab stock as a result of crowding, improper diet, poor culture maintenance or disease and acclimation time from field to laboratory conditions.

These conditions may also result in irreproducibility of results during repeated testing of a particular chemical. Variation in lethality to wild populations of invertebrates after repeated toxicity testing may suggest the need for using a reference stock of invertebrates. The reference stock may be used as a standard to compare sensitivity or health of other invertebrate stocks. Variations in the sensitivity of the reference stock may be determined by using a reference chemical.

Another problem of acute invertebrate bioassays is length of a bioassay. Situations such as delayed mortality (Hubschman, 1965) may occur. Frequently *Hyalella* or snails may remain in severe stress for hours or days.

Time of death is often difficult to determine when invertebrates are in severe stress. Defining time of death has been a problem of invertebrate toxicologists. Lack of visible response to continued prodding is generally accepted. In some cases if the organism is exposed to a red light cessation of heart or intestine movements may be termed as death.

Toxicity of various contaminants to fish and invertebrates is variable (Table 1). To obtain a true indication of the effect of a contaminant on the aquatic environment several species of the food chain must be exposed, since fish or invertebrates may be more susceptible to a particular toxicant. Comparatibility of results cited in the literature is often difficult to assess. This is due to variation in the physiological condition of the organism, dilution water chemistry or toxicant composition.

Acute lethal testing may not always be a good measurement for comparing toxicity of a contaminant to fish and invertebrates. Additional information may be obtained using sublethal bioassays such as activity (Wallace et al., 1975) and avoidance (Costa, 1966) experiments. In future studies results from the combination of acute and sublethal studies may be a more accurate indicator of fish and invertebrate sensitivity to a toxicant.

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Development of a Field Bioassay Using *Daphnia pulex*

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ABSTRACT

The relative sensitivity of 3 species of fish and 15 species of invertebrates was tested using various concentrations of a reference mixture of toxicants. The reference toxicant mixture contained soft water, kaolinite (20 mg/l), ammonium chloride (10 mg N/l), No. 2 fuel oil (10 mg/l) potassium chromate (0.25 mg Cr/l), sodium monosulfide (0.17 sulfide/l) and phenol (0.10 mg/l). Expressed as fractions or multiples of all the above concentrations, the 24 hr lethal concentrations of this mixture for 50 percent of the exposed organisms were *Daphnia pulex* (0.11), *D. magna* (0.16), *Aedes aegypti* (2.3), *Nitocris* sp. (3.7), *Salmo gairdneri* (4.0), *Hyallela azteca* (5.6), *Dero* sp. (5.6), *Tubifex* sp. (5.6), *Goniobasis* sp. (7.4), *Stylaria* sp. (6.0), *Philodina acuticornis* (6.0), *Dugesia tigrina* (6.3), *Lepomis macrochirus* (7.4), *Helisoma* sp. (7.4), *Cambarus* sp. (7.5), *Carassius auratus* (7.8), *Aeolosoma headleyi* (8.3), and *Physa* sp. (9.2).

Daphnia pulex was selected as a candidate screening organism for field bioassays of refinery effluents because it was 1) the most sensitive of all animals tested, 2) relatively inexpensive, 3) relatively easy to maintain in the laboratory, and 4) because it is a potential fish-food organism in many unpolluted waters of North America.

A simple static bioassay using *Daphnia pulex* was evaluated in three ways. First, animals from three cultures were tested with the reference toxicant mixture over a three month period. Second, field bioassays were conducted by refinery personnel at six different locations using the reference toxicant mixture. Results were consistent among the refineries. Finally, duplicate field bioassays of refinery effluent gave similar results.

INTRODUCTION

Most bioassay procedures currently used to monitor effluents are cumbersome and expensive in terms of the required time, facilities, technicians and test organisms. Finding invertebrates that are as sensitive as, or more sensitive, than fish and the development of appropriate bioassay techniques may allow more rapid and economical testing of industrial effluents. Although selecting an organism "sensitive" to all kinds of pollution is virtually impossible, it seemed worthwhile to attempt this for a specific category of pollutants. Many invertebrates are more easily cultured than fish and have a shorter life span. In some instances invertebrate test organisms are more easily maintained. The use of more sensitive organisms to screen effluents is necessary as more stringent limits are placed on effluents.

The purpose of this project was the development of a miniaturized, simple and inexpensive bioassay method that could be used for evaluating toxicity of petroleum refinery effluents. To achieve this objective an array of freshwater invertebrates and fish were evaluated with special attention to commercial availability, sensitivity to toxicants, and ease in handling by people with little or no biological training. This bioassay method was then tested in ways that would help in evaluating it. Finally, refinery personnel were trained to use the bioassay method and they tested it at various refineries.

This work was sponsored by the American Petroleum Institute. A more complete description of this work may be found elsewhere (Buikema, et al., in press).

METHODS

The evaluation of test organisms was based on several criteria: (1) the organisms had to be easy to observe; (2) the bioassay had to be relatively easy to perform; (3) the organisms had to be easy to handle; and (4) special training and equipment had to be kept to a minimum. Another important consideration was keeping the space required for acclimation and testing reduced to a minimum and if possible to produce results in as short a time period as possible.

Effluent limitation guidelines for petroleum refineries have been suggested by the United States Environmental Protection Agency for major contaminants in refinery effluents for 1977. Because there is no "typical" effluent and because complex industrial effluents change during storage, we chose to work with an Arbitrary Reference Mixture (ARM) made with specific chemicals (Table 1). This mixture was prepared without the oil component at 100 times the concentrations shown here. Test solutions were made by diluting the 100 X stock solution with dechlorinated tapwater. Oil was added to the test solutions with a micropipet. The resulting mixture was multiphasic, having oil on top, dissolved and suspended matter in solution and sediment on the bottom.

In order to find a suitable test organism the reference mixture was used to screen 15 freshwater invertebrates and 3 species of fish. These animals were collected from the field or obtained from commercial suppliers. The invertebrates were acclimated to lab conditions in dilution water for at least 3 days prior to the toxicity test. Fish were acclimated for at least a week before use. Light intensity and photoperiod were controlled. Temperature was 21 ± 2 degrees Celsius. All tests were conducted without aeration and without renewal of test solution.

RESULTS

The array of freshwater organisms shown in Table 2 were tested. The pulmonate snails, Physa and Helisoma, were the least sensitive of all animals tested. The river snails, Nitrocris and Goniobasis, were somewhat more sensitive. Cladocerans, both of them Daphnia species, were the most sensitive of all animals tested.

Tubifex worms were quite sensitive, but to observe them it was necessary to pull clumps of them apart and this may have stressed them thus increasing their

TABLE 1. Basic formulation of the Arbitrary Reference Mixture

Parameter	Concentration	Ingredient
NH_4-N	10. mg/l	NH_4Cl
Cr	0.25 mg/l	K_2CRO_4
Oil and grease	10.	No. 2 fuel oil
phenol	0.10 mg/l	phenol
sulfide	0.17 mg/l	$Na_2S \cdot 9H_2O$
TSS	20. mg/l	kaolinite
pH	6.8-7.2	$NaOH/H_2SO_4$

TABLE 2. Comparative tolerance of selected freshwater invertebrates and fish exposed to the Arbitrary Reference Mixture (ARM). LC$_{50}$ values are expressed as multiples and fractions of the ARM shown in Table 1.

	24 HR LC$_{50}$
Gastropoda	
Physa sp.	9.2 - 22.0
Helisoma sp.	7.4
Nitrocris sp.	3.7
Goniobasis sp.	5.6 - 7.4
Crustacea	
Amphipoda	
Hyallela azteca	5.6
Cladocera	
Daphnia pulex	0.11
Daphnia magna	0.16
Decapoda	
Cambarus sp.	7.5
Oligochaeta	
Dero sp.	5.6
Tubifex sp.	1.8 - 5.6
Stylaria sp.	6.0
Aeolosoma headleyi	8.3
Turbellaria	
Dugesia tigrina	4.9 - 6.3
Rotifera	
Philodina acuticornis	6.0
Insecta	
Aedes aegypti	2.3
Osteichthyes	
Salmo gairdneri	4.0
Lepomis macrochirsu	7.4
Carassius auratus	7.8

apparent sensitivity. Mosquito larvae 24 to 48 hrs old at the start of the test gave LC_{50} values of 2.3. Mosquito larvae exposed to the reference mixture within an hour or two after hatching had 24 hr LC_{50} values of only .5 to .8. This was probably due in part to the fact that young mosquito larvae seemed more likely to get caught in the surface film.

The fish species tested -- rainbow trout, bluegills, and goldfish -- were insensitive to the reference mixture. In fact, they were roughly comparable to some of the snails. Five of the invertebrates tested were more sensitive than the rainbow trout.

Table 3 shows the response of representative forms for 24, 48 and 96 hr exposures. For our purposes, the mosquito and the cladocerans were judged suitable for industrial toxicity tests. The studies on the mosquito were discontinued because use of the species required a permit and because in the public view, mosquitoes are considered a nuisance.

Daphnia were the most sensitive of the animals tested and were equally easy to observe and handle in the lab. They also are important fish food organisms in many unpolluted waters of North America. After 24 hrs exposure, the *Daphnia* were 70 times more sensitive than goldfish. *Daphnia* species have been used for testing toxicity since 1929 (Berger). There is some literature to suggest that the response of *Daphnia* is less variable than the response of fish (Macek and Sanders, 1970) and at least one paper has judged *Daphnia* to be representative of other predominant zooplankton (Anderson *et al*. 1948).

D. pulex was selected instead of *D. magna* for three reasons: (1) *D. pulex* is distributed over the entire North American continent while *D. magna* is restricted to the more northern and western regions of North America; (2) *D. magna* is typically

TABLE 3. LC_{50} values for selected organisms after 24, 48 and 96 hr exposure. LC_{50} values are expressed as multiples and fractions of the ARM shown in Table 1.

	LC_{50} Values		
	24 h	48 h	96 h
Nitocris	3.7	1.9	1.8
Hyallela	5.6	3.2	1.3
Daphnia	.10	.07	.03
Aedes	2.3	--	1.8
Carassius	7.8	7.0	6.4

a hardwater species whereas D. pulex is found in a wide range of habitats; and (3) the cosmopolitan distribution is D. pulex may be important to regulatory agencies that specify animals for toxicity testing.

Daphnia were reared in soft, carbon-dechlorinated tapwater. The Daphnia were fed 24 hrs prior to use with a fine suspension of trout chow. The animals were collected with a coarse net (0.6 to 1.0 mm mesh) which was moved slowly through a culture tank. Any rapid seining with the net caused many animals to become floaters. To avoid trapping air beneath the carapace, the net was not removed from the tank completely. After about three minutes essentially all of the small, immature Daphnia swam out of the cone-shaped net.

A medicine dropper with a large bore (1.5 mm) was used to transfer the larger Daphnia to the test containers. The animals were added to the dilution water by placing the tip of the medicine dropper beneath the surface film.

Death, defined as cessation of all visible signs of mobility of the second antennae, respiratory appendages and the post-abdomen, was used as the endpoint. If no movement was observed after 5 seconds, the animal was considered dead. All movement was observed with the unaided eye or with a magnifying lens.

Twenty-two bioassays were conducted with the reference mixture. Results are shown in Table 4. The least variability occurred after 48 hr exposure. Twelve tests were conducted with the reference mixture without the oil component (Table 4). Oil definitely has an effect on the LC_{50} values and much of its effect was due to surface entrapment. Similar effects of oil have been observed by others (Grodner, 1957; Dowden, 1965). However, even without oil, the reference mixture was toxic enough to give an LC_{50} value of less than 1 after 48 hours exposure.

The results of refinery tests using the ARM in dechlorinated tapwater are shown in Table 5. In comparing refinery results with ours, the mean data were quite similar after 48 hours.

TABLE 4. Mean and standard deviation of LC_{50} values obtained for Daphnia pulex bioassays of the ARM and of the ARM without oil. LC_{50} values are expressed as multiples or fractions of the ARM

REFERENCE MIXTURE

hour	mean LC_{50}	-SD	+SD
8	.18	.1	.33
24	.11	.06	.42
48	.07	.04	.11
96	.03	.02	.04

REFERENCE MIXTURE
WITHOUT THE OIL COMPONENT

hour	mean LC_{50}	-SD	+SD
8	3.84	2.93	5.02
24	1.76	0.99	3.13
48	0.96	0.76	1.20
96	0.42	0.27	0.66

TABLE 5. Mean and standard deviation of LC_{50} values obtained for bioassays of the ARM of our laboratory and at different refineries.
LC_{50} values are expressed as multiples or fractions of the ARM.

REFERENCE MIXTURE

hour	Mean LC_{50} Our Lab	Mean LC_{50} Refinery Lab
8	.18	.48
24	.11	.27
48	.07	.13
96	.03	.05

Reproducibility among the various laboratories was less than reproducibility at each laboratory but this was expected. Possible reasons for variability among the refinery laboratories were: 1) most participants or their technicans had little experience in doing bioassays; and 2) refinery tests were performed using *Daphnia* that had undergone the rigors of cross-country shipping. There was also some difficulty in adding small but exact amounts of oil to the test containers.

The effluents of the refineries involved in the "round robin" testing program exhibited a wide range of characteristics. Table 6 shows the monthly averages for the effluents at refineries where bioassays were conducted. Refinery D had the highest concentrations of chromium, oil and grease, phenols, and sulfide. Refineries E and F had high concentrations of oil and grease. The ARM (Table 1) contained moderate concentrations of all toxicants compared to the actual effluents shown in Table 6.

The results of *Daphnia* bioassays using refinery effluents are shown in Table 7. Most bioassays were done in duplicate and the LC_{50} data were from eye-fitted curves. Each pair of numbers represents the results of duplicate tests on the same effluent sample. There was little variation between the LC_{50} values for replicate tests indicating that reproducibility was possible with this procedure.

Several features of the *Daphnia pulex* bioassay should be improved. The present method of using the entire population from a culture tank involves some risks. Old, senile individuals may be included in the test groups and as a result the condition of the culture tank may bias results. Results of recent studies suggest that rigid requirements (such as use of early instars, narrow temperature limits, or absence of ephippial eggs in the culture tank) may not be absolutely necessary for a screening test useful in testing toxicity of effluents.

TABLE 6. Monthly averages of effluents at refineries where toxicity tests were performed. All values are expressed as mg/l except pH

Parameter	REFINERY					
	A	B	C	D	E	F
ammonia-N	5	69	12	23	7	7
chromium	.005	.08	.06	1.5	-	-
oil & grease	3	4	5	53	14	11
pH	8.2	7.7	7.2	8.4	7.4	7.2
phenols	.01	.02	.04	8.9	.1	.01
sulfide	2	.05	0	1.9	0	.1
suspended solids	19	8	36	66	66	27

TABLE 7. *Daphnia* bioassays using refinery effluents. 48 h LC_{50} values are expressed as percent effluents.

Test	REFINERY A	B	C	D	E	F
1 a	C	NT	18	5.6	9.4	88
b	C	93		6.0		NT
2 a	NT	NT	3.8	5.6	2.8	NT
b	NT	NT		9.8		84
3 a	NT	NT	C	3.2	2.3	89
b	NT	NT		3.2		100
4 a	NT	NT	5.0	1.2	1.4	56
b	NT	NT		1.3		43
5 a	13	NT	--	1.0	1.0	66
b	17	NT		1.4		62

C = could not determine

NT = not toxic to 50% of the population

We are currently analyzing data pertaining to the effect of photoperiod, light intensity, temperatures and culture condition on results of the *Daphnia* test. In the months ahead we will be testing refinery effluents to determine the degree of correlation between *Daphnia* and fish bioassays.

CONCLUSIONS

1. The bioassay method for on-site testing of refinery effluents shows promise but further development is needed.
2. After 24 hrs exposure to the reference mixture, *Daphnia pulex* was more than 40 times as sensitive as rainbow trout and 70 times as sensitive as goldfish.
3. The *Daphnia* bioassay requires less space and time than the fish bioassay.
4. The *Daphnia* bioassay results are reproducible and appear suitable for screening refinery effluents.

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LOBSTER AND OTHER DECAPOD CRUSTACEAN LARVAE AS TEST ORGANISMS IN MARINE ACUTE TOXICITY BIOASSAYS*

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ABSTRACT

Certain decapod crustacean larvae have been simply and effectively used as test organisms in marine acute toxicity bioassays in the laboratory. Decapod larvae fit some of the criteria of a "standard" or "recommended" test organism. Lobster larvae probably best fulfill the criteria in Eastern Canada at the present time, as they are available, culturable, amenable to bioassays, comparatively well studied, and sensitive to pollutants. Hatching and culture techniques, and bioassay procedures including response criteria, are described. Use of lobster larvae for regularly-scheduled acute bioassays is considered labor-intensive. This problem notwithstanding, lobster larvae could be considered a candidate for a "standard" or "recommended" marine plankter for aquatic toxicity laboratories. The more frequent use of decapod larvae generally as test organisms is encouraged.

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1 INTRODUCTION

In Canada, pulpmill and refinery effluents, municipal sewage effluents, dredging spoils, mine tailings, and various other chemical wastes continually enter coastal waters (Waldichuk, 1974). Most of the standard bioassay procedures incorporate freshwater organisms, but because of marked physiological differences between freshwater and marine organisms, there is a continual need for bioassays utilizing sensitive life stages of marine invertebrates and fish. Waldichuk (1973) intensively reviewed the methodologies of marine bioassays and supported comparative studies using several kinds of aquatic organisms. Such studies identify the acute toxicities of marine contaminants and their components at or close to source and investigate under controlled conditions the biological problems identified or suspected in the field.

Marine organisms being considered as standard bioassay organisms should fulfill five major criteria (Sprague, 1970; Waldichuk, 1973; Buikema _et al._, 1974; Maciorowski, 1975; Stephan, 1975):

1) Availability-locally, readily and in large numbers, at low cost.

2) Culturable in laboratory; feeding possible.

3) Amenable to acute lethal-sublethal tests and chronic sublethal tests.

4) Importance ecologically and commercially. Well-known biology.

5) Sensitivity to unnatural stressors.

This paper discusses how larvae of the American lobster, _Homarus americanus_, fulfill these criteria, with some limitations, and briefly summarizes other studies with decapod larvae and aquatic pollutants, with the general aim of encouraging more frequent use of these organisms in marine bioassays.

2 LOBSTER LARVAE AS TEST ORGANISMS

2.1 Availability

Hatching times can be controlled and large numbers of larvae are available at each hatch. With careful planning, larvae of Homarus americanus can be made available in a laboratory nearly year-round, at low cost beyond manpower requirements. This has already been accomplished in several Canadian and U.S. laboratories.

Mature female lobsters extrude eggs in early to late summer; the eggs are fertilized during extrusion and are carried on the pleopods for approximately ten-twelve months (McLeese & Wilder, 1964) (Fig. 1A). The number of eggs per female varies from 3,000 to 100,000 (Barnes, 1911; Herrick, 1911; Mackay, 1929; McLeese and Wilder, 1964) and is directly related to size of the lobster (McLeese and Wilder, 1964). With permission of fisheries protection personnel, females with new eggs can be collected from coastal waters in the Maritimes in late summer or early fall, independently or through local suppliers or fishermen. Lobster maintenance and egg development has been well described (Smith, 1873; Bumpus, 1891; Herrick, 1911; Davis, 1964; McLeese and Wilder, 1964). Hatching the eggs retained throughout development by the female lobster is recommended (Rice and Williamson, 1970). Hatching times can be controlled, egg development being related to both water temperature and their age when subjected to different thermal conditions (Templeman, 1936a; Perkins, 1972). Hence, a continual and abundant supply of larvae is possible over 8 to 12 months of the year from an initial supply of 30-40 adults. Eggs from one lobster hatch over approximately two weeks at 20 C and more slowly at

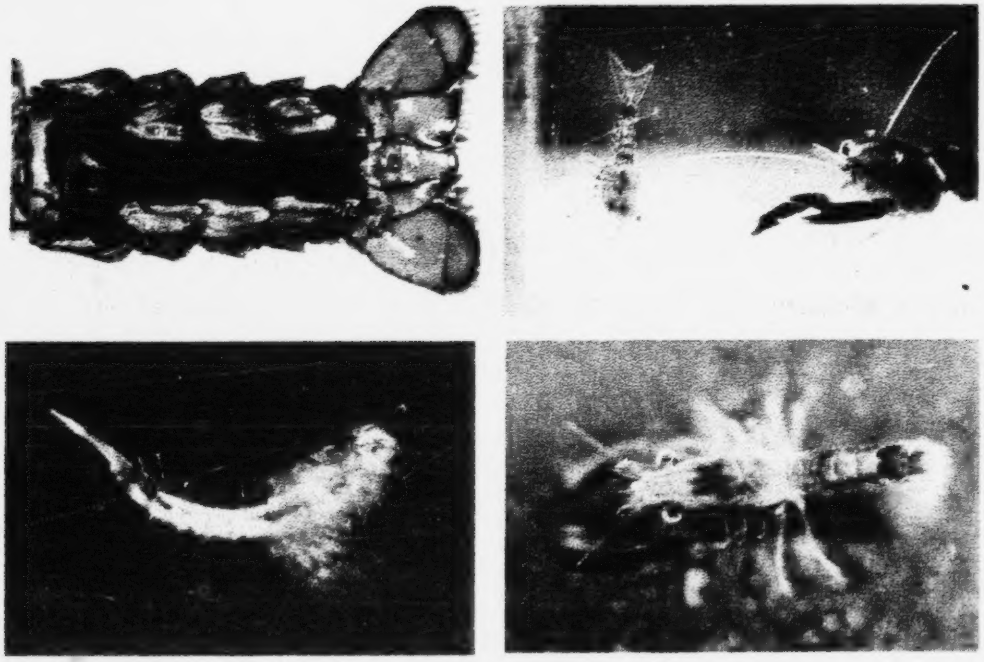

Figure 1. A. Ventral view of abdomen of "berried" female lobster; B. Larval stages one and four in an aquarium (courtesy of J.Mitchell, Woods Hole); C. Incapacitated stage one larva, paralysed and incapable of swimming; D. Stage four "intermediate" larva, showing shortened antennae and visible external thoracic exopodites.

lower temperatures (Herrick, 1911; Hughes and Matthiessen, 1962; McLeese and Wilder, 1964). Minimum hatching temperatures were 9.4 C and 5-6 C (Bardach *et al.*, 1972; Wells, unpubl. data), followed by normal larval development. Eggs should be hatched in tanks designed to keep newly-hatched free-swimming larvae from being lost with outflowing water. Larvae should be removed from the hatching tank soon after hatching and be well fed with freshly-hatched *Artemia salina* nauplii. Larvae hatching at different times should be kept separate in well-aerated vessels to enable larvae of known age and from a similar position on the egg mass to be incorporated into a single experiment.

Once hatched, larvae develop rapidly to fourth larval stage (in 13-14 days at 19-20 C, and in 3-4 weeks at 15-16 C, according to Hughes and Matthiessen (1962), and in 10-14 days at 20 C in studies by J. Castell (pers. comm.)). Hence they must be quickly used when in the required stage. Because of variable egg survival and development and/or unexpected events, the immediate availability of newly-hatched larvae may fluctuate from time to time throughout the year.

2.2 Culture

A vast literature exists on the culture of lobster larvae and post-larvae (Scattergood, 1949a,b; Lewis, 1970; Nowak, 1972). This section describes how larvae can be cultured prior to and during acute and chronic bioassays with pollutants.

Larvae have often been reared successfully for experiments (Fig. 1B); rearing temperatures were about 20 C, water was static for single larvae or flowing for mass cultures; larvae were generally fed natural plankton and more recently, brine shrimp nauplii. Larvae have also been reared by aquaculturists in continuous-flow and recirculating mass culture systems, with survival to the fourth stage ranging from 22-85% (Bardach et al., 1972; Hughes et al., 1974; Serfling et al., 1974a). Many factors, including food, the occurrence of fouling or disease organisms, and conditions of salinity and temperature, determine the chances of successful culture. Besides *Artemia* nauplii, best culture foods, in particular for larvae in mass culture, are ground clams, frozen and live adult brine shrimp and crab viscera (Bardach et al., 1972; Serfling et al., 1974b; Castell, pers. comm.). Survival to fourth stage in "Hughes" recirculating tanks was best with *Artemia* nauplii (Hughes et al., 1974). However, constituents for a nutritionally correct diet for larval culture have not yet been determined but are being investigated (Castell, pers. comm.). In recent culturing efforts, cannibalism, gas disease and metal ions were major problems (Bardach et al., 1972); infestations of ciliates and a bacterial disease have also been observed (Aiken et al., 1973; Serfling et al., 1974c).

In a study investigating the toxicity of crude oil dispersions (Wells and Sprague, 1975) the bioassay technique was static, with no aeration, larvae being placed singly into jars holding 800-825 ml treatment solution at a depth of 15 cm. Jars were maintained at 20 C. Assignment of larvae to each treatment and jars to a tank was randomized. Transparent lids prevented entry of air-borne dust and water. Each larva was fed 500-1000 *Artemia* nauplii daily. Treatments were totally changed every four-five days in longer-term (30 day) experiments. A 14L:10D photoperiod was maintained using fluorescent lights, 0.5 to 2 m above the treatment jars. Measured light intensity at surfaces of containers was 25-50 foot-candles. Salinity, oxygen, pH and temperature were regularly monitored. Larvae were successfully studied in 4 day and 30 day bioassays using these simple procedures.

In summary, most hatches of lobster larvae are amenable to culture and feeding. Larvae are small (approx. 7-8 mm long when hatched) and only require small (half to one litre) holding and testing containers. If freshly-hatched larvae are required, there is a short acclimation time to water temperature and characteristics. However, larvae must be tested individually in static bioassays, to avoid cannibalism and to prevent food loss. Treating animals individually increases information per test animal but significantly increases labor, especially in chronic bioassays. Studies are required to determine if continuous-flow bioassays could be conducted with multiple units containing single larvae, with food being introduced intermittently, so as to reduce this labor.

2.3 Bioassay Procedures

Bioassays with lobster larvae should be conducted following the principles in Sprague (1969, 1973) and Perkins (1972a, b), with a consolidation of the above techniques of larval culture. Hence, test containers are inspected at times from one hour to four days at approximate logarithmic intervals, and daily if tests are continued beyond four days. In the study by Wells and Sprague (1975), data were recorded in numerical code on Fortran sheets, facilitating data retrieval and analysis.

Some of the responses measured in this bioassay study (Wells, 1975) were as follows:

a) Incapacitation - A larva was incapacitated if it was prostrate in the test container, but alive (heart beating), with appendages moving (Fig. 1C). An ET50 (median effective time) was estimated using methods of Litchfield (1949).

b) Death - A larva was dead if, upon microscopic examination, no heart beat was present, although appendages could still be moving slightly. LT50's (median lethal times) were estimated using methods of Litchfield (1949). LC50's (median lethal concentrations) were estimated by either methods of Litchfield & Wilcoxon (1949) or with Fortran program BMD03S (Dixon, 1970). The stage of the molting cycle of the lobster larva in which mortality occurs, whether it be in A to D (between molts) or in E (during molt), based on Aiken (1968), should be noted.

c) Determination of larval stage/occurrence of cast shell - If a larva had molted, the developmental stage was determined using characteristics given by Barnes (1911), Herrick (1911) and Templeman (1936a, 1948). Stages one to four are easily separable by eye, based on criteria of morphology and size. Cast shells were examined microscopically to verify the normal morphology of the previous stage. The presence of a cast shell also verified the occurrence of molting. The absence of a cast shell did not necessarily mean that molting had not occurred since the last observation, as most casts are eaten soon after the successful molt. In longer-term (30 day) experiments, the rate of development of the larvae through to stage four was determined, based on daily records on the larval stages.

d) *Observations on morphological variations* - Once larvae were in stage three, larvae and their cast shells were particularly examined to observe normal development of stage three larvae to stage four. "Intermediate" fourths occassionally occur at this time (Templeman, 1936b; Wells, 1972; Wells and Sprague, 1975) (Fig. 1D).

e) *Growth* - The carapace and total lengths of the surviving fourth and fifth stage larvae were measured using an ocular micrometer.

f) *Larval behaviour* - Position in water column, swimming pattern, feeding and defecation rates, and color were described or measured.

Specific considerations to the use of lobster larvae as bioassay test organisms are:

a) *Certain developmental stages* are preferable for short-term (4-day) acute lethal bioassays. Stage one larvae suffer a high natural mortality rate at molt. To avoid this problem and to avoid molts during the short-term bioassays, use of stages two, three and four are recommended, stage three probably being the preferred fully planktonic stage in acute bioassays at 20 C as it spends 6-7 days in stage three at this temperature.

b) *Mortality among control larvae* often occurs in 4-day tests, especially if larvae molt during the test. Assuming independence between the causes of this mortality and that occurring due to treatments, a correction can be applied to the data using Abbott's formula (Tattersfield and Morris, 1924). Such mortality is unfortunate but appears typical of some invertebrate bioassays (Sprague and McLeese, 1968; Maciorowski, 1975).

c) *Larvae are generally fed during the bioassay*, due to their rapid weakening when starved (McLeese, pers. comm.). Hence, there exists the potential for pollutant intake via food, or during course of ingesting food, as well as through other sites of entry i.e. gills, carapace. Food intake is not constant as it is influenced by both molting cycle and food density. Larvae should be well-fed prior to bioassays to ensure their optimum baseline condition.

In summary, larvae are amenable to both acute and chronic bioassays, combining lethal and sublethal responses. They are small organisms, facilitating many treatments in minimum space. Specific points relating to their use as test organisms are the use of specific developmental stages, the occurrence of control mortality and the necessity to feed larvae during bioassays. Bioassay personnel can be relatively easily trained to culture larvae and conduct acute bioassays. However, culture, feeding and use of larvae in regularly-scheduled acute bioassays is considerably more labor intensive than similar procedures with standard fresh water organisms, such as the rainbow trout (*Salmo gairdneri*).

2.4 Information on Biology

Lobsters are important ecologically (Mann & Breen, 1972) and commercially (Wilder, 1970). Because of the long importance of the lobster fisheries and lobster culture in northeastern North America there is a relatively large literature on the larvae, compiled most recently by Nowak (1972).

At this time, there are laboratory programs using lobster larvae and post-larvae regularly in toxicity bioassays (D.W. McLeese at St. Andrews, N.B.), in nutrition and growth studies (J. Castell, Halifax, N.S.), and in aquaculture studies (Aiken _et al._, St. Andrews, N.B.; G. Jameson, Halifax, N.S.; Ford _et al._, San Diego, California, U.S.; Hughes _et al._, Martha's Vineyard, Mass., U.S.). The aquaculture studies are a potential source of larvae for testing purposes, as has already been demonstrated by Hughes and J. Castell (pers. comm.).

2.5 Response to Pollutants

Based on a comparison of acute lethal toxicities, lobster larvae are most sensitive to an insecticide (fenitrothion), metals, dispersed crude oil and an anionic detergent and least sensitive to quartz particles and a bleached kraft pulp mill effluent (Table 1). Lobster larvae are sensitive zooplankters; a level of protection for them might result in protection for other species at their trophic level in salt water, and for more resistant species at other trophic levels.

TABLE 1. Summary of acute lethal toxicities, expressed as 96 hr LC50's (medium lethal concentrations), of various aquatic contaminants to lobster larvae, in order of decreasing sensitivity.

Order of Sensitivity	Contaminant	LC50, mg/l	Reference
1	Insecticide - fenitrothion	0.001	McLeese (1974)
2	Metals - Hg - Cu^{+2}	0.03-0.1* 0.1-0.3*	Connor (1972) Connor (1972)
3	Sodium Lauryl Sulphate	0.72	Wells and Sprague (1975)
4	Ven. Crude Oil	0.86	Wells and Sprague (1975)
5	Non-ionic Dispersant	46	Engel and Neat (1971)
6	Oil Refinery Effluent	1000-3000	Wells (1975)
7	Quartz Particles	No lethality at 5000-10,000	Cobb (1972)
8	Bleached Kraft Mill Effluent	No lethality below 100,000	Sprague and McLeese (1968)

* 48 hr LC50, with *Homarus gammarus*.

3 DECAPOD LARVAE IN GENERAL

There is considerable information on larval decapods that have now been studied in culture in the laboratory, most such studies having concentrated on positive identification of species and detailed morphological descriptions and having been encouraged by contributions of Costlow et al. at Duke University, Provenzano (1967) and Williamson (1967). There is a recent trend towards the study of culture techniques, and physiological and ecological requirements of larvae (Rice and Williamson, 1970; Sastry, 1970; Sulkin and Minasian, 1973; Sandifer, Smith and Calder, 1974, among others).

This trend has resulted in a number of toxicity studies with decapod larvae (other than lobster larvae, treated in previous section), summarized in Table 2. These studies have mainly examined acute lethal effects of toxicants, in short-term tests. Decapod larvae have not been considered suitable for longer term toxicity testing because of labor intensive static culture and poor survival of controls. Most studies have used crab and lobster larvae, shrimp and prawns seldom being tested. Multichambered aquaculture trays or single vessels, with static test solutions, are most commonly used; continuous-flow methods continue to be developed (Buchanan et al., 1975). Besides lethality, developmental rates, morphology, behavioural patterns and various physiological responses have been studied in tests lasting from a few days to a few weeks. The relatively few toxicity tests to date emphasize the sensitivity of decapod larvae to aquatic pollutants, especially chlorinated hydrocarbons, metals, dispersed oils and detergents, and emphasize the growing interest in their culture for bioassays.

TABLE 2. A summary of the types of pollutants and response parameters examined in toxicity bioassays with decapod larvae, excluding lobster larvae.

Organism		Pollutant	Response Parameter						Reference
			Surv.	Devel.	Morph.	Behav.	Accum.	Phys.	
Shrimp	*Crangon*	$HgCl_2$	+						
		$CuSO_4$	+						Connor (1972)
	Penaeus	L. Crude	+						Anderson *et* *al*. (1974)
Crabs	4 spp.	DDT	+		+				Bookhout and Costlow (1970)
	Leptodius	Dieldrin	+	+			+		Epifiano (1971, 1972, 1973)
	3 spp.	Mirex	+	+			+		Bookhout *et* *al*. (1972, 1975)
	Cancer	Sevin	+	+		+			Buchanan *et* *al*. (1970)
	Carcinus	$HgCl_2$	+						Connor (1972)
		$ZnSO_4$	+						
		$CuSO_4$	+						
	Uca	$HgCl_2$	+			+		+	DeCoursey and Vernberg (1972) Vernberg *et* *al*. (1973)
	Cancer	No. 2 Fuel	+						Vaughan (1973)
	Neopanope	Ven. Light Crude	+	+					Katz (1973)

4 SUMMARY AND CONCLUSIONS

The responses of numerous decapod crustacean larvae to toxicants are now being studied, both in the laboratory (see section 3) and in the field (DeCoursey and Vernberg, 1975; Gabriel et al., 1975). Decapod larvae fit some of the criteria of "standard" laboratory test organisms. Epifiano (1971) suggested that crab larvae be investigated as to their use as bioassay organisms. Lobster larvae probably best fulfill the criteria in Eastern Canada at the present time, as they are available, culturable, comparatively well studied and sensitive to pollutants. However, use of lobster larvae for regularly-scheduled acute bioassays is labor intensive and relatively specialized. Continued improvements on present culture and bioassay test systems may reduce this labor but the special methods required may, as with oyster embryos (Stephan, 1975), preclude the common acceptance of lobster larvae as routinely used organisms. These problems notwithstanding, lobster larvae could be considered a candidate for a "standard" or "recommended" marine plankter for aquatic toxicity laboratories.

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COMPARISON OF SENSITIVITY BETWEEN THREE INVERTEBRATES (*CHIRONOMUS*, *GAMMARUS*, *AEDES*) AND RAINBOW TROUT TO KRAFT MILL EFFLUENT

by

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COMPARISON OF SENSITIVITY BETWEEN THREE INVERTEBRATES (*CHIRONOMUS*, *GAMMARUS*, *AEDES*) AND RAINBOW TROUT TO KRAFT MILL EFFLUENT

Fish bioassay, as was proposed by Doudoroff (1951), is being used here in Canada, as well as in most of the other countries of the world, as the standard method for the testing of the toxicity of industrial liquid wastes. This standard method determines the mean lethal (or tolerance) limits of a toxic substance and can be taken as an index of the relative toxicity of this substance to the fish tested. However, this measure cannot indicate, with any certainty, the concentration which is expected to be safe or harmless to the various organisms in the aquatic community.

Recently, the need was felt for the development of other bioassay techniques which would be sensitive enough to measure the effects of deleterious substances on a larger segment of the biological community. The development of a more comprehensive bioassay, where several other organisms can be used, will be greatly appreciated by those who may not be able to obtain easily and at all times the one or few fish species defined in a standard bioassay procedure.

In selecting test organisms other than fish, researchers are usually looking for smaller and simpler organisms which can be obtained easily or maintained inexpensively under laboratory conditions. These organisms have to be sensitive enough to respond to low concentrations of the deleterious materials, and there should be a simple way of monitoring their responses. It is also preferable to find organisms which are known to constitute important links in the food chain of the aquatic community.

The present paper describes briefly the results of a research program sponsored by the Committee on Pollution Abatement Research and executed by BEAK in Toronto. The objective of that research was to evaluate the sensitivity of three invertebrate organisms known to be important in the food chain to treated Kraft mill effluent, and compare their sensitivity with that of rainbow trout which is used here in Ontario and widely on the Federal level as a standard test organism .

The three invertebrates tested were the midge *Chironomus tentans*, the amphipod *Gammarus pseudolimnaeus*, and larvae of the mosquito *Aedes aegyptii*. The organisms were reared in the laboratory under controlled temperature and light conditions and their life cycle was observed carefully by a qualified biologist.

The bioassays were conducted in small beakers, using the various life stages of the organisms. The effluents used in these bioassays were actual Kraft mill effluents, treated by various procedures. However, due to the time limitation of this presentation, only the results of the tests using primary treated Kraft mill effluent will be discussed. For comparison, the sensitivity of the rainbow trout towards the same effluent was also measured during this investigation.

Table 1 shows the sensitivity of the various stages of the aquatic organisms tested towards the primary treated Kraft mill effluent. The results indicated no significant differences between the sensitivity of the 2nd and 3rd instar of *Chironomus* and its more advanced 4th instar, while there were some differences between the young stage of *Gammarus* and the mature stage. The juvenile stage was more sensitive, particularly at high levels of effluent concentration.

Figure 1 is a graphic representation of the data in Table 1. The sensitivity of the organisms in this figure was defined as being physiological because, during the test, the organisms were subjected to the effluent in glass containers in the laboratory, which does not represent by any means the ecological conditions of these organisms in their natural habitat. This is particularly true for the *Chironomus* which lives in tubes in the mud substrate. During this test, the *Chironomus* larvae were taken out of their tubes and placed in glass beakers. Figure 1 indicates that the physiological sensitivity of *Chironomus* to the effluent was greater than that of trout, while the sensitivity of *Gammarus* was less than that of the *Chironomus* or the trout. *Aedes*, on the other hand, was the most resistant organism.

In order to investigate the effect of the presence of mud substrate on the sensitivity of *Chironomus*, another set of test vessels was prepared, containing a thin bottom layer of mud substrate. *Chironomus* larvae were introduced into these containers and were subjected to a 65 percent concentration of the effluent. The results of this test are shown in Figure 2. It is obvious from the figure that the larvae, when protected by the mud substrate, were less

Table 1
Sensitivity of Various Aquatic Organisms towards Primary Treated Kraft Mill Effluent.

ORGANISM	PERCENT SURVIVAL AFTER 96-HOUR EXPOSURE						
	Effluent Concentration (% by volume)						
	0%	10%	20%	40%	65%	80%	100%
Rainbow trout	100	100	80	40	0	0	0
Chironomus							
(2nd & 3rd instars)	100	90	70	0	0	0	0
(4th instar)	100	100	60	0	0	0	0
Gammarus							
(juvenile)	100	100	100	60	40	0	0
(mature)	100	100	100	70	70	60	50
Aedes							
(1st instar)	100	100	100	100	100	100	100
(4th instar)	100	100	100	100	100	100	100

Figure 1
Physiological Sensitivity of Aquatic Organisms to Primary Treated Kraft Mill Effluent.

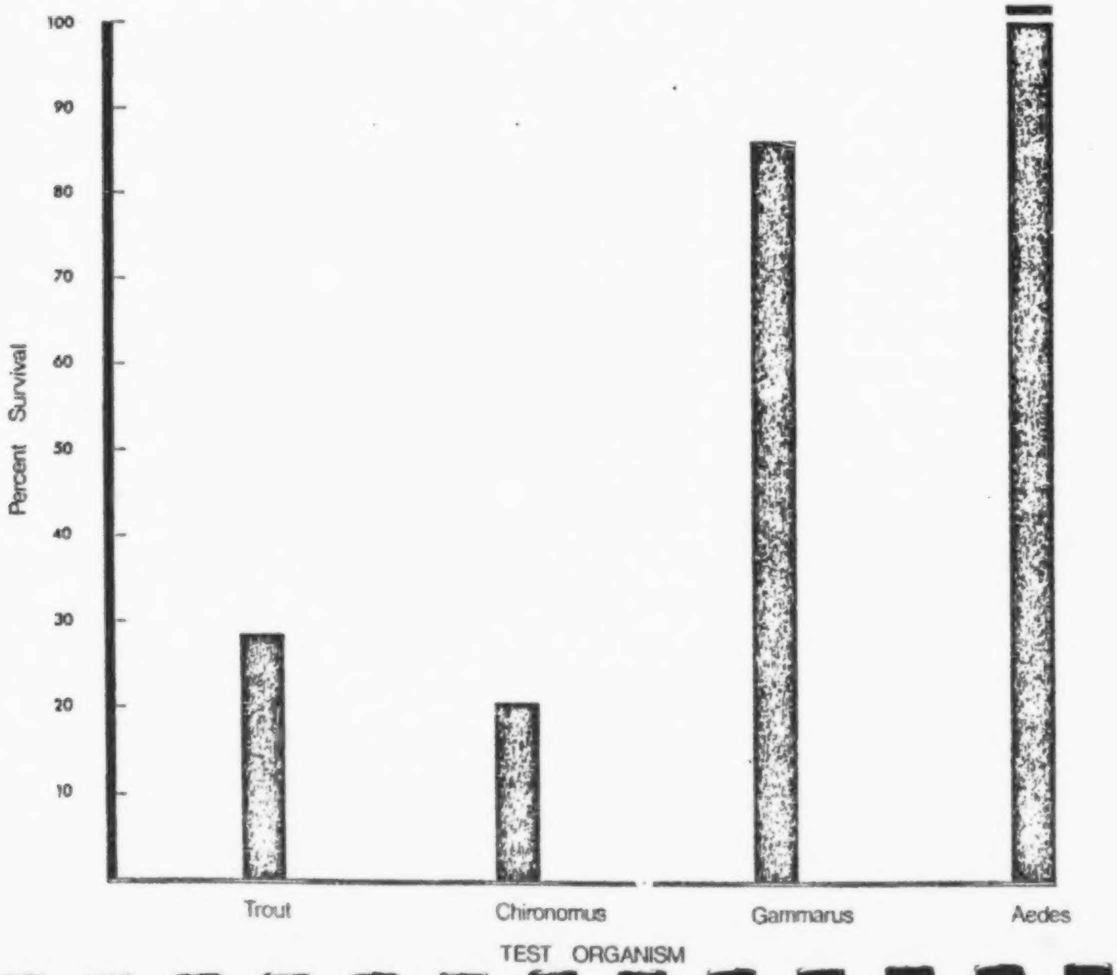
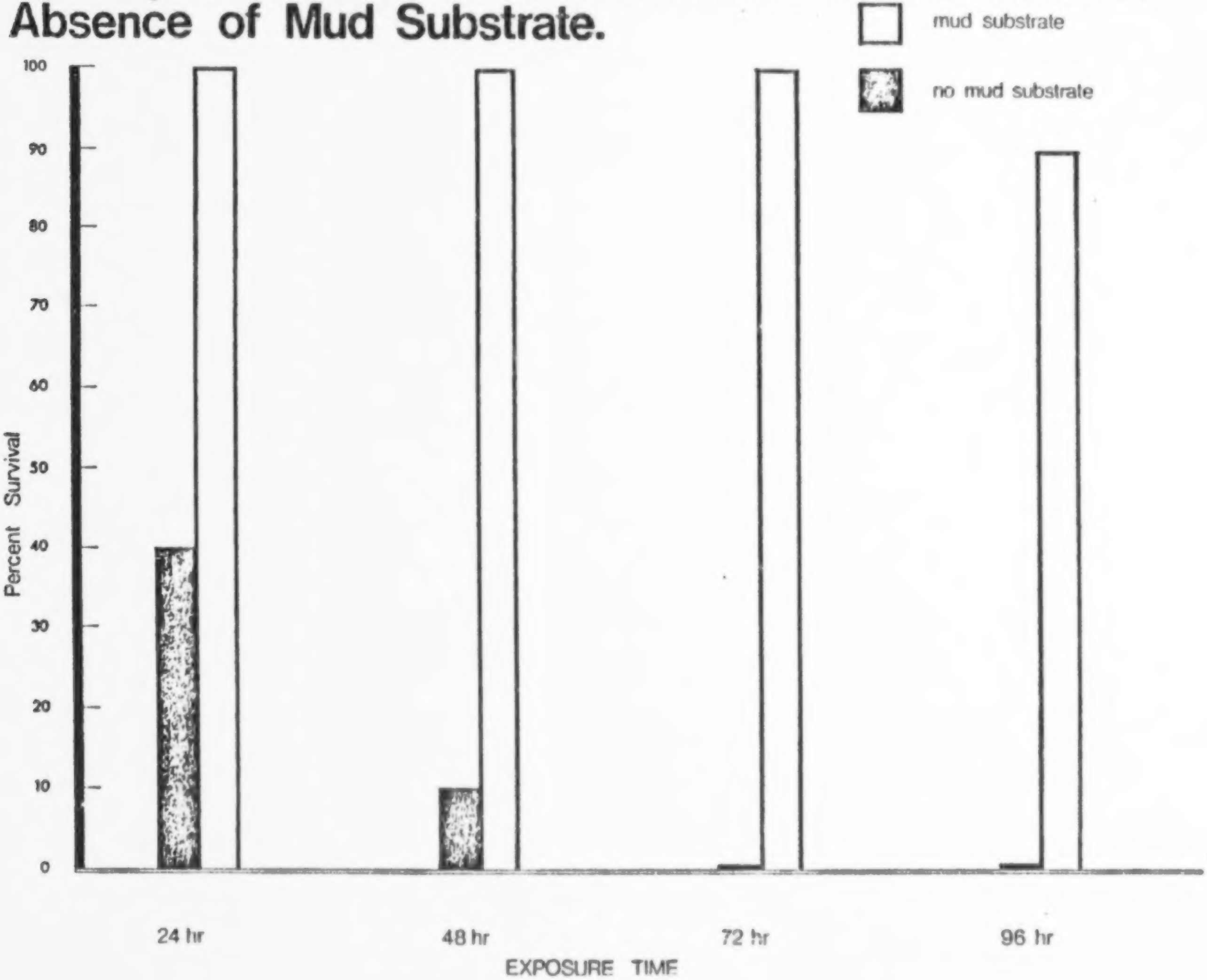


Figure 2
Sensitivity of Chironomus to 65% Concentrations of Primary Treated Kraft Mill Effluents in Presence or Absence of Mud Substrate.



sensitive to the toxic effluent. There is a great possibility that some of the toxic substances were adsorbed by the mud surface. This would have been the case under natural ecological conditions. This could explain why ecologists usually consider Chironomids as pollution tolerant species.

Figure 3 represents the relative ecological as opposed to physiological sensitivity of the free swimming fish and *Gammarus* and the *Chironomus* when protected by the mud substrate. The time of exposure to the effluent was extended during this experiment to seven days. It is apparent from Figure 3 that the sensitivity of the organisms varied with time. Within the first 24 hours of exposure, the fish seemed more sensitive than the other organisms tested. However, during the following 3 days, *Gammarus* started to be affected by the toxicants and it became more susceptible to them than the other organisms during the last period of the experiment.

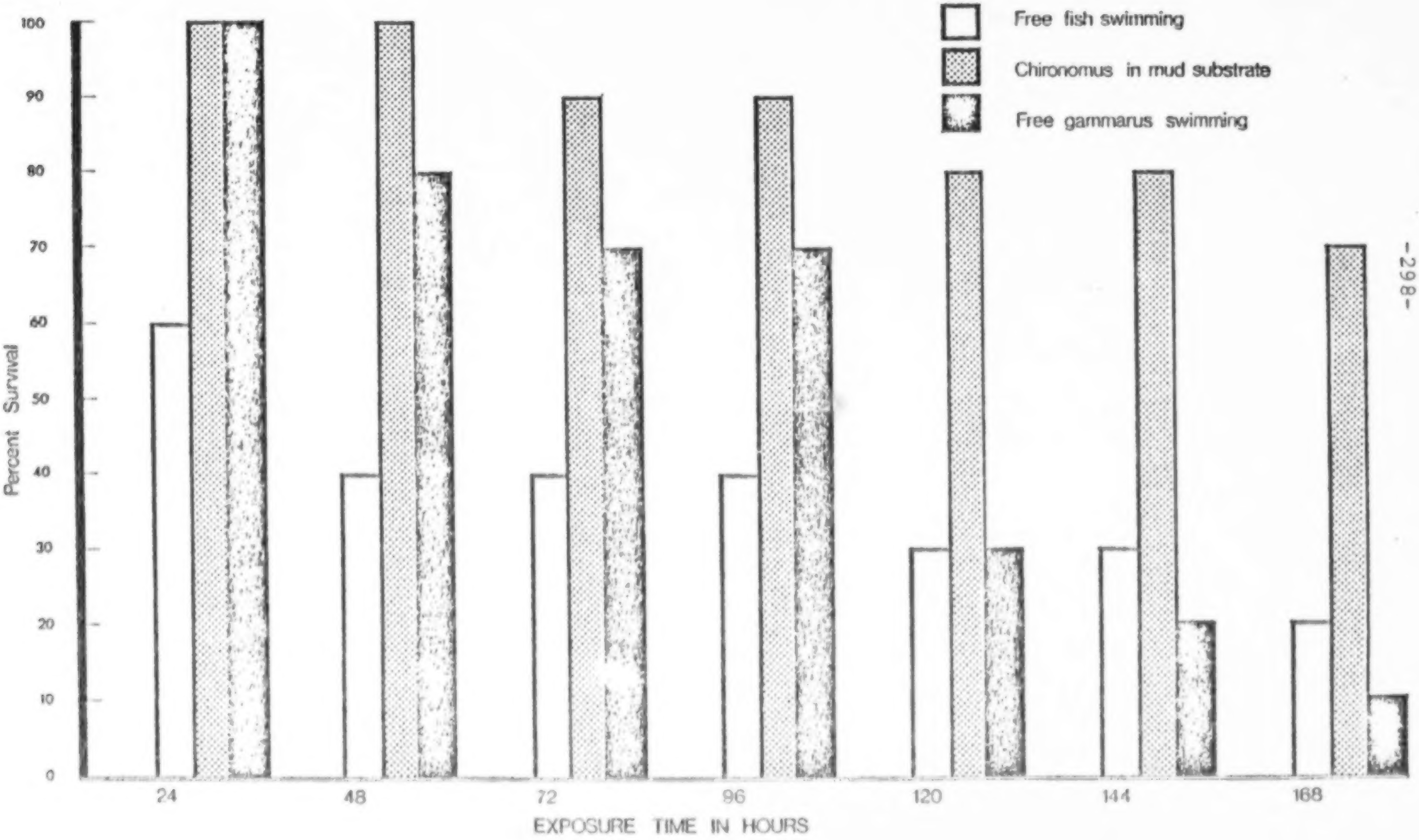
In conclusion, it may be safe to assume that during our search for alternative organisms for bioassays, we should consider the following:

1. The stage of the life cycle of the organism to be used in the test should be easy to identify and maintain under the desired temperature and light conditions.

2. In the determination of a measure of sensitivity, it may be wise to distinguish between the response of the organisms in the laboratory and its expected response when in its natural habitat.

3. A correlation between the sensitivity of the new species and that of a conventionally used standard organism should be established for future reference. Standard toxicants, such as dodecyl sodium sulphate or any other suitable material can be used in the establishment of such a correlation.

Figure 3
Relative Ecological Sensitivity of Aquatic Organisms to 40 % Concentrations of Primary Treated Kraft Mill Effluent



THE CULTURE OF HYALLELA AZTECA
FOR EXPERIMENTAL USE

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The Culture of Hyallela azteca for Experimental Use

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Expanded Abstract:

Hyallela azteca, a common fresh-water amphipod was studied with the intent of defining conditions which controlled reproduction and conditions which optimized the yield and health of the animals. The studies were done with several common problems of invertebrate bioassays in mind- unsolved problems such as the reproducibility of results, constant control mortality, and explanations for inconsistencies in results.

Light cycles of 12L:12D and of shorter light, and light intensities of approximately 12 $\mu E\ m^{-2}\ sec^{-1}$ and dimmer, terminated reproduction at temperatures between 10°C and 25°C. The same 12L:12D cycle, but increased from a shorter one, and light intensities of 12 $\mu E\ m^{-2}\ sec^{-1}$ and brighter, initiated reproduction at all temperatures between 16°C and 26°C. Lower temperatures were not tested. Temperature had a strong influence on the rate and intensity of both responses. At temperatures lower than 20°C more Hyallela went into diapause significantly faster, and came out of diapause significantly more slowly, than at temperatures greater than 20°C. Also, more animals responded to the increased 20L :4D cycle at temperatures above 20°C than at lower temperatures.

Animals matured at temperatures less than 20°C are larger than those matured at warmer temperatures. In the wild, these large animals which mature in late fall go into diapause. However, large size does not cause diapause, nor does diapause cause an increase in adult body size.

Preliminary toxicity tests and other mortality data show that culture conditions are important to Hyallela's response under stress. Conditions under which the animals mature are probably more important than the conditions to which they are acclimated in the laboratory,

providing they are not subjected to drastic stresses. Field populations constantly change in population structure, and in the size and "health" of adult animals, hence these are not a good source for bioassay animals. Also, these population parameters may vary considerably from one lake to the next.

In view of all the results obtained, it was decided that imposing a cycle which includes a time for diapause and a time for reproduction is the best strategy for the culturalist who is interested in uniform animals. This strategy can be used to synchronize reproduction and then to grow animals to the desired stage. The mixed population structure which is typically found in bodies of freshwater can be avoided.

The results will be published in more detail by the author within the next year. One paper will be entitled "Factors affecting the initiation and termination of diapause, and size relationships associated with diapause, in the freshwater amphipod *Hyallela azteca*". Another will be a technical report on general culture methods.

GAMMARUS CULTURE USING DECHLORINATED WATER

by

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INTRODUCTION

The successful culture of aquatic organisms in the laboratory may depend on the similarity of laboratory conditions with that of the organism's biological requirements. The following outline deals with the specific problem of culturing the chlorine sensitive organism *Gammarus pseudolimnaeus* Bousefield (Arthur and Eaton, 1971) in a dechlorinated municipal water supply.

Holsinger (1972) reported that all life-stages of *Gammarus* were available in the field year round. However, there was a greater seasonal abundance of specific life stages observed in the field-sampled streams. Personal observations supported the annual breeding cycle theory of Hynes and Harper (1972). It was felt therefore that laboratory cultures of *G. pseudolimnaeus* would provide a more homogeneous bioassay stock of known age on a regular basis throughout the year and a better biological understanding of the amphipod. The criterion for success was that the culture methodology should be capable of producing 360 invertebrates weekly to supply three bioassays of six concentrations each containing 20 *Gammarus*.

Although this study is of a preliminary nature, an attempt is made to assess the economics of culturing *G. pseudolimnaeus*.

The average total chlorine residue (T.C.R.) level maintained in the municipal water at the point of consumption was 300 to 500 µg/litre T.C.R., (Metropolitan Toronto Works Department, 1975). The municipal water was passed through activated charcoal before entering the laboratory. The laboratory's dechlorinated water had about 30 µg/litre T.C.R., (Ontario Ministry of the Environment (MOE) Water Quality Section, 1975). The T.C.R., shown in Table 1, consists of free and combined available chlorine. Mono- and di-chloramines were the major components of the T.C.R. (MOE Water Quality Section, 1975).

Arthur and Eaton (1971) found the T.C.R. (as chloramines) 96-hour LC_{50} for *G. pseudolimnaeus* to be 220 µg/litre and the chronic 15 week no effect level measuring reproductive impairment to be 3.4 µg/litre. Therefore, the need to develop a non-chemical water treatment system to further reduce the T.C.R. level to a non-detectable level was also realized in

Table 1: Total Chlorine Residue

Free Available Chlorine:

$Cl_2 + H_2O \quad H + Cl^- + HOCl$ (Hypochlorous acid)

$HOCl \quad H + OCl^-$ (Hypochorite ion)

Combined Available Chlorine:

$NH_4 + HOCl = H + H_2O + NH_2Cl$ (Monochloramine)

$NH_2Cl + HOCl = H_2O + NHCl_2$ (Dichloramine)

$NHCl_2 + HOCl = H_2O + NCl_3$ (Trichloramine)

Plus organic associated chloro-derivatives.

Table 2: Total Chlorine Residual Removal at Different Flow Rates Through the Charcoal-Ultraviolet Treatment System

Flow Rate (litre/minute)	Total Chlorine Residue (µg/litre)	Monochloramine (µg/litre)	Dichloramine (µg/litre)	% Removal
Initial	28	9	18	0
1.0	0	0	0	100
1.5	5	0	5	82
2.5	6	0	6	80
6.5	11	0	11	61

this study. T.C.R. reductions in water supplies has been accomplished by other investigators using activated charcoal and sodium thiosulphate (Smith, 1973) and ultraviolet irradiation (Armstrong and Scott, 1974).

METHODS

Parental stocks of G. pseudolimnaus were collected from the Credit River near the Forks of the Credit and from Shelter Valley Creek near Centreton, Ontario. Groups of 25 Gammarus were placed in three litre glass jars and held in a water bath at the stream temperature for two days. They were then acclimated to 17°C (Smith, 1973) at the rate of one Celcius degree per day (Sprague, 1963).

All cultures were fed partially decomposed elm and maple leaves as recommended by Kaushik and Hynes (1971). Watercress, live Daphnia sp. and chopped earthworms supplemented the diet. A 16-hour light photoperiod was maintained at 14-20 footcandle illumination by two 30 watts, wide spectrum, "Duro-Test" fluorescent tubes. Water changes of the parental cultures with dechlorinated laboratory water were arbitrarily established at the rate of 50% replacement every two weeks.

Gravid female Gammarus, identified by enlarged brood pouch development, were isolated in 600 ml beakers until the release of their young. Each beaker contained a pre-soaked leaf and watercress. The young were collected in glass capillary pipettes and counted. The females were returned to the static culture containers.

The culture conditions for the growth of the young Gammarus were identical to those of the parents. In addition the young received some particulate leaf matter collected from the adult static jars and were allowed to feed on the algae that grew on the side of the culture tanks.

A single group of newly released Gammarus, numbering 400-800 organisms, were raised under each of the following water treatment conditions.

1) Untreated Static I

The young were placed directly into dechlorinated laboratory water and held under static conditions in 3 litre containers which were aerated continuously. The water was replaced every two weeks with fresh, dechlorinated, laboratory water.

2) Treated Static II

Dechlorinated laboratory water was aerated for 24-hours prior to the introduction of the young, continuously aerated thereafter and replaced every two weeks with 24-hour, pre-aerated, laboratory water.

3) Treated Static III

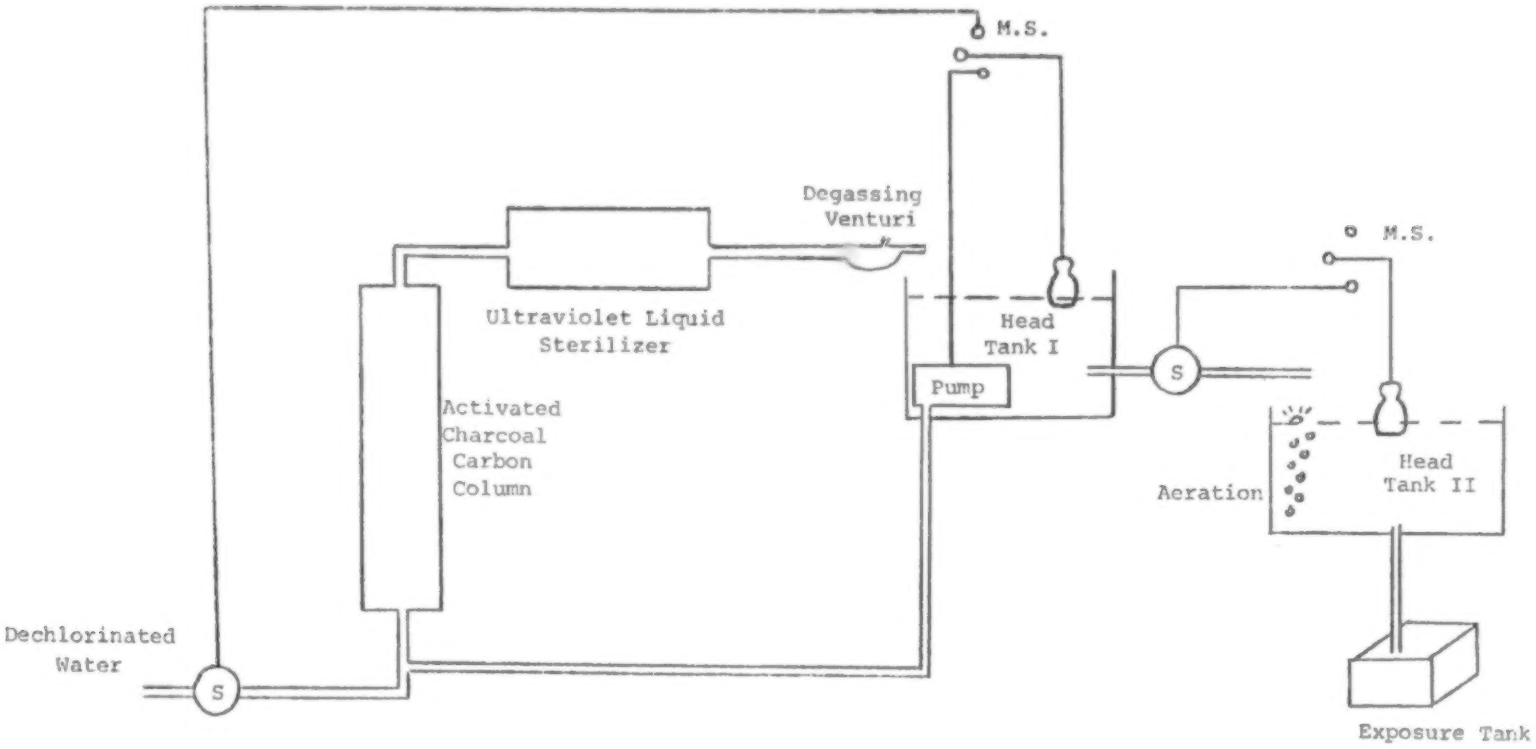
Dechlorinated laboratory water was aerated for 24 hours prior to the introduction of the young, aerated continuously thereafter and replaced every three weeks with 24-hour pre-aerated laboratory water.

4) Untreated Continuous Flow I

Dechlorinated laboratory water continuously flowed at the rate of 0.5 litres per minute into 10 litre aquaria containing the young *Gammarus*.

5) Treated Continuous Flow II

Dechlorinated laboratory water was passed through an additional activated charcoal column (cross-sectional area: 322 sq. cm. by 1.8 meters depth), through a single-tube 50-watt ultraviolet sterilizer (Aquafine SL-1) and finally through a venturi to fill a 700-litre constant level head tank at 1 litre per minute (Figure 1). When the head tank was filled the treated water was recirculated through the charcoal-ultraviolet system. The head tank drained into a second 700 litre constant level head tank which was vigorously aerated and fed into individual aquaria through separate tubes at the rate of 0.5 litres per minute.


M.S.
Degassing Venturi
M.S.
Ultraviolet Liquid Sterilizer
Head Tank I
Pump
S
Activated Charcoal Carbon Column
Aeration
Head Tank II
Dechlorinated Water
S
Exposure Tank

M.S. - Microswitch (SPDT)

S - Solenoid

FIGURE 1: WATER TREATMENT EQUIPMENT FOR TOTAL CHLORINE RESIDUE REMOVAL

Gammarus were not exposed to the "treated continuous flow" system as a separate case but those organisms held under "treated static II" conditions were transferred to the "treated continuous flow" system after 60 days. They were then allowed to grow to maturity and produce a second generation.

Exposure to the above water treatments continued for 90 days or until 100% mortality was observed. Mortality was recorded at 30, 60 and 90 days.

The T.C.R. analysis which consisted of free and combined available chlorine were conducted by the MOE Water Quality Section utilizing an amperometric-polarographic method. Three litre volumes of dechlorinated, laboratory water were analysed after various aeration times. The continuous-flow, dechlorinated, laboratory water was analysed at different flow-rates through the charcoal-ultraviolet treatment systems.

Cesilum chloride (99.9% purity) was used to preliminarily compare the sensitivity of adult *G. pseudolimnaeus* cultured in the laboratory with those collected from the field. The first generation laboratory cultured adult *Gammarus* were raised from parental stocks collected from the Credit River. The field collected adult *Gammarus* were collected from the Credit River sampling site and Duffin Creek near Pickering, Ontario. Each bioassay included six logarithmic concentrations of Cs^+ with each concentration containing 20 amphipods. The 96-hour LC_{50} values were estimated according to Sprague (1973). Their corresponding confidence limits were calculated according to Litchfield (1948). The dissolved oxygen, pH and test temperatures were stable in all the tests at 9.2 ± 0.5 mg/litre, 7.9 ± 0.2 and 17.0 ± 1°C respectively. The organism lengths were 8.0 ± 1.2 mm.

RESULTS

The T.C.R. analysis of the aerated water indicated that the monochloramines were undetectable after two hours while the dichloramines were undetectable after 18 hours, Figure 2. Similar analysis of the water after a single passage through the charcoal-ultraviolet treatment system indicated that a flow of 1 litre per minute or less removed all chloramines, (Table 2).

Survival of the young Gammarus held under "untreated static I conditions" decreased during the first 60 days with complete mortality occurring after 90 days, (Table 3). The "treated static II" conditions resulted in 32% mortality after the first 60 day period while the "treated static III" conditions resulted in 100% mortality after 60 days exposure.

The "untreated continuous-flow I" group died within 30 days while the "treated static II" group transferred to the "treated continuous-flow II" conditions suffered only 3% mortality during the first 30 days after transfer. The later group continued to develop and produce a second generation after 5-7 months.

Thirty-five females from the parental culture stock required 18.7 ± 2.5 days at 17°C for the full development of their eggs and an average of 21.7 ± 9.3 young were released. The young Gammarus were about 4 mm and suitable for bioassay use after 90 days. Death sizes of the 52 adults from the parental culture stock that died while exposed to the untreated, continuous-flow water for 60 days were 12.9 ± 2.2 mm. To eliminate the moribund stage of field collected adults for bioassay experimentation by size restriction, those amphipods between 6 to 10 mm should be used.

The cesium chloride test results are shown in Table 4. The 96 hr. LC_{50} concentrations of Cs^+ for the laboratory cultured, Credit River and Duffin Creek amphipods were 37, 45 and 64 mg/litre respectively.

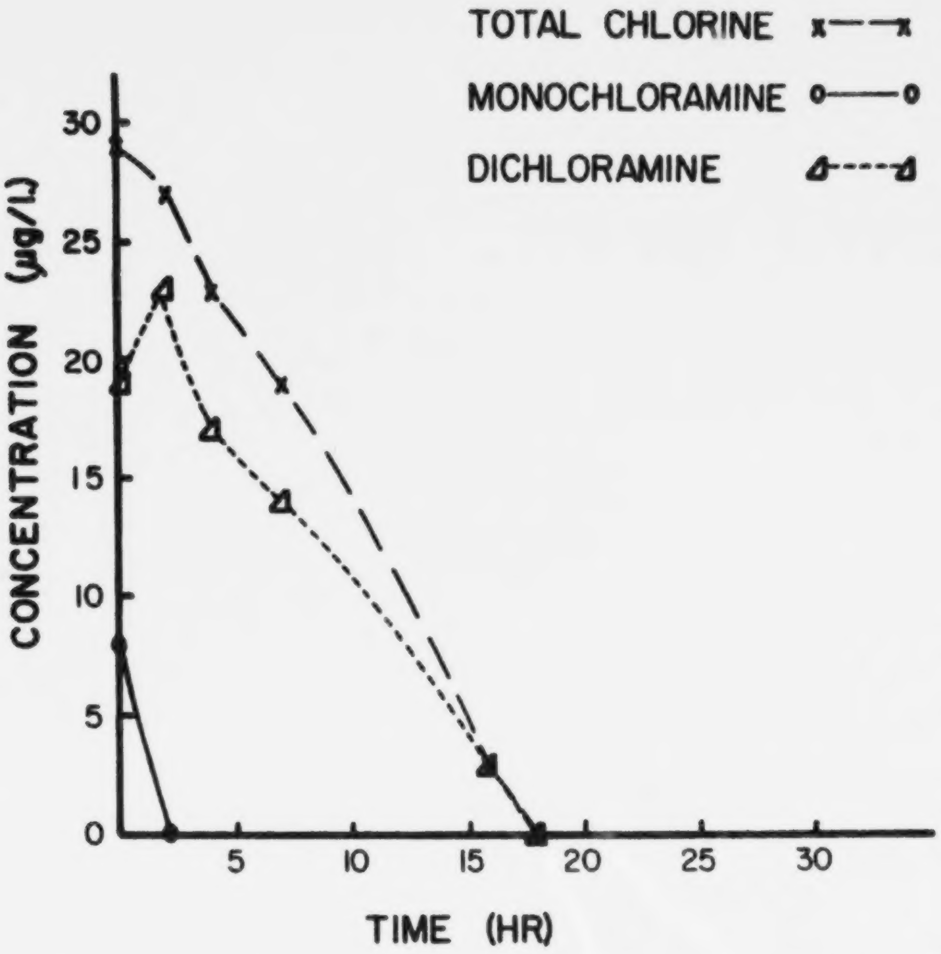


Figure 2: Aeration Time Required to Reduce the Total Chlorine Residue, Mono- and Dichloramine Levels to N.D. in Three Litre Static Volumes of Water.

* N.D. - nondetectable

Table 3: Mortality of the Young *Gammarus* Exposed to Different Laboratory Water Treatments

Water Treatments	No of Organisms	Percent Mortality		
		30 days	60 days	90 days
Untreated Static I	800	59	72	100
Treated Static II	510	21	32	-
Treated Static III	420	27	100	100
Untreated Continuous-flow I	640	100	100	100
Treated Continuous flow II	344	-	-	35

Table 4: Cesium Chloride Bioassay Test Results

Bioassay No.	Sample Location	96 hr. LC_{50} (Log-Probit Estimate)	96 hr. LC_{50} Confidence Limits
1	Laboratory Culture	39 mg/litre	30-51 mg/litre
2	Laboratory Culture	36 mg/litre	28-45 mg/litre
3	Credit River	45 mg/litre	32-63 mg/litre
4	Duffin Creek	60 mg/litre	47-76 mg/litre
5	Duffin Creek	68 mg/litre	47-99 mg/litre

DISCUSSION

The sensitivity of the young Gammarus to the chlorine residue, as discussed by Arthur and Eaton (1971), was substantiated with the survival of the young being inversely related to the T.C.R. levels.

The "untreated static I" group would have been subjected to various increases of the T.C.R. levels, to about 30 µg/litre, every two weeks. Although the levels became undetectable after 18 hours the periodic exposures were above the recommended 3.4 µg/litre level of Arthur and Eaton (1971) and could be responsible for the 100% mortality of the young Gammarus within the 90 day period. The most successful group, "treated static II" was essentially never exposed to the T.C.R. and experienced 32% mortality over 60 days. The high mortality in the "treated static III" conditions was unexplainable. The mortality in the "untreated continuous-flow I" condition was fairly rapid due to its continuous exposure to 30 µg/litre of T.C.R. The "treated continuous-flow II" water maintained the "treated static II" group until maturity. It would appear that even the later 60 day age class would still be relatively chlorine sensitive since the "untreated static I" group suffered the remaining 28% mortality (to total 100% mortality) during the last 30 days.

Assuming that a minimum of 11 young would be produced after a 21 day incubation period at 17°C by each female and assuming 35% mortality over 90 days, the stock requirements to maintain a continuously producing culture can be determined.

In order to supply 360 organisms weekly, 51 gravid females would have to be selected to produce young each week and three groups would have to be established, each to produce on a consecutive week. An equal number of males should be provided for the 150 females to ensure a reasonable mating frequency. The young would be about 4 mm in length, an adequate size for bioassay use, after 90 days which would necessitate a three month time-lag before the first generation young would be available. Five to seven months would be required before sexual maturity would be reached and the second generation produced.

These organisms would not be available for bioassay use for an additional 90 days.

It is evident that a laboratory culture of G. pseudolimnaeus would require considerable time to establish. A more viable alternative would be to collect and hold field-specimens.

The 96 hr. LC_{50} and their confidence limits of the Cs^+ sensitivity tests indicate minor variations between the laboratory cultured amphipod, (cultured from the Credit River parental stocks), and the Credit River field-collected amphipods.

Although the 96 hr. LC_{50} and their confidence limits seem to indicate a greater difference in response between amphipods of different watersheds the difference appears insignificant. Any geographical variation in response could be avoided by collecting the specimens from a single watershed.

The continuous-flow system for supplying cultures and dilution water for bioassays can prove to be a labour saving approach compared to managing static cultures and preparing dilution water by aeration. A larger unit for practical purposes would have to be constructed to provide the flow characteristics required of a large culture collection and the associated, bioassay, dilution water requirements. The described unit could produce only one litre per minute of treated water at peak, operational performance.

Due to the extensive time required for the production of successive generations of Gammarus pseudolimnaeus a more fecund invertebrate should be chosen for reproduction studies.

ACKNOWLEDGEMENTS

I am grateful to the Ministry of the Environment's Water Quality Section for performing the chemical analysis.

Note: Mention of commercial trade names does not imply endorsement by the Ministry of the Environment.

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DISCUSSION OF

INVERTEBRATE BIOASSAY AND CULTURE

John Sprague

I would like to ask the assembled experts which is the better invertebrate for reproductive studies, is it Gammarus pseudolimnaus, Daphnia or Hyallela?

Bridget de March

Daphnia has the problem of ephippia eggs and I think it has even more drastic size differences than Hyallela. I have had a lot of trouble inducing the second generation of Gammarus to reproduce. Gammarus collected in the fall or summer will always reproduce, but I am not doing something right for the next generation. Hyallela is the easiest at this stage.

David Lee

As far as ease of culturing is concerned, one should consider Daphnia. D. pulex would be better for reproductive studies because it requires only one month to complete a life cycle while D. magna requires six months.

John Davis

I have a philosophical point that someone might like to comment on. It would appear that we now have a standard fish bioassay using a salmonid. I would like to hear what we should be doing about invertebrate bioassays, are there species both in fresh and salt water that can stand alone as toxicity testing organisms?

Dr. Fahmy

I do believe that bioassays should be more versatile than being restricted to just one test organism. I know several institutes and industries that would like to use rainbow trout but are not able to obtain the fish. Once they obtain them they cannot maintain them. The idea of having more than one organism that can be used in a bioassay is a good idea. In order to compare the results of one test to another we have to standardize our tests and I believe if we establish a correlation between some of the invertebrate organisms and a standard organism such as the rainbow trout, then we can relate the results of any test to a specific organism with a limited amount of certainty.

Bob Cook

I have heard of a lot of candidates to replace rainbow trout. We should be expanding on all the biological information. It should not be one versus the other but I think there should be a degree of purpose involved with these tests. The organism that will give you the answer and perspectives for that question should be the organism of choice. I think the subject of the rainbow trout test at least relates to the legislation "Deleterious to fish". A decision was made that the rainbow trout will be that fish for a time frame.

There are three basic areas that we have to take into consideration. One is a regulatory responsibility, the second is really the ecological implications and how to best study things in the environment and the third area is the advancement of scientific knowledge. Purpose should be the first lesson and we should not really be saying one species is better because we can raise it better than another species.

Bridget De March

The big advantage of using invertebrates is simply the accumulation of data. More data can be generated in less time using invertebrates rather than fish. Cost and space requirements will also be reduced using invertebrates.

Bob Cook

Legislation says "deleterious to fish" and that is how you make your impact. A million Hyallela will not make a point but one dead rainbow trout will.

Peter Wells

The objectives of a particular bioassay should be carefully thought out before setting up any sort of program and this runs into the same area on which Bob Cook has just commented. The other point is that the best bioassay organisms are obviously those that have been studied for some time. You have to have some sort of information base to work from rather than attempting to work with and culture organisms whose biological requirements are not well understood. Regulatory agencies are further ahead by using an animal which has been well studied when they approach industry. The lobster larvae are great animals to use when dealing with specific problems on the east coast yet they are not very meaningful to people on the west coast.

TOPIC VI

REFERENCE TOXICANT TESTING

Open Discussion - Reference Toxicant Testing

Cecil Inniss

We have by no means a definite format for this session, but I was hoping it would prove to be the most useful and practical session in that there is a certain lack of focus as a result of workshop meetings. The result presentation is one thing but they rarely effect some change in the way that we are doing things. I propose to invite three gentlemen, plus myself who would act as a panel on reference toxicant testing. Hopefully we will formulate a list of people who have laboratories working in the field, who feel themselves capable to conduct a reference toxicant test with one of the proposed chemicals.

There has been some discussion here this afternoon about the various philosophies of bioassay testing. One of the things that tends to be overlooked is that frequently we are not talking of the fine things we sometimes get into arguments about. Some of the fish I have seen in bioassays look more like question marks than fish, some are positively cadaverous around the stomach. These are things that are quite obvious, but nevertheless people seem to allow these fish to find their way into bioassay jars. The problem will be intensified now as a result of the federal guidelines and the participation in toxicity testing by industries who do not always have the manpower or are not willing to put manpower into what is essentially a non profit and rather expensive business. We cannot involve ourselves in a long-term program to upgrade toxicity testing without a certain financial commitment but I think there are certain bootstrap efforts we can make to obliviate these shortcomings. If we try to coordinate our efforts in referenc toxicant testing we may find that there are certain hatcheries that produce fish that are not suitable for our purposes.

I have no intention of remaining non-polar in this discussion because I feel it is impractical to use reference chemicals which have a rather doubtful formulation, are difficult to analyse, or which require extensive gas chromatographic verification. I think it would be better to stick to a fairly simple chemical, so as to aid people who do not have the multi-million dollar laboratories backing them up.

I chose cesium chloride as a reference toxicant since it was a polar compound, easily soluble, and readily available. Unfortunately it turned out that cesium chloride was not terribly toxic. I could not persuade flagfish to die at 560 ppm. It costs $25 for 25 grams and needless to say, if it is not very toxic and if it costs a lot nobody is ever going to want to test it, particularly in continuous flow.

Jack Klaverkamp, John Davis and Ed Pessah, I gather there is a difference in opinion about the type of reference toxicant to be used or whether a reference toxicant should be used at all.

Jack Klaverkamp

I think it has all been said, why use it? Inter-laboratory comparisons, we agree are perhaps secondary to having some internal control system within a laboratory. I know that pharmaceutical companies test mice, rats or the standard laboratory animal once in a while with some reference standard toxicant to ensure the population is representative. We are not trying to protect the individuals, but populations. I think the idea of reference toxicants is philosophically on sound ground. Perhaps the fault is that we read too much into it. I agreed with John Davis that they should be used to detect large differences and not to the exclusion of physical testing.

Cecil Inniss

John Davis, would you please defend the use of dehydroabietic acid and sodium pentachlorophenate.

John Davis

I did not really use those two substances but I thought they were the be all and end all of reference toxicants. We happened to be doing a lot of work with pulp mill wastes and it seemed wise to test dehydroabietic acid because many of the seven laboratories involved in these intercalibration studies were using a substance present in an industrial waste that was being widely tested in the region. You have eluded to some problems with dehydroabietic acid and I would agree with you there are several, primarily, the source.

Probably a few good chemists in the country could synthesize it but not in large quantities and it is quite toxic. Secondly quantification of its presence is necessary and requires sophisticated chemistry. Thirdly, it has its problems with solubility. We found in testing for the availability in bioassay tanks, there was only 85% of the theoretical concentration actually present. Long-term continuous flow tests as opposed to static bioassays can reduce the threshold toxicity considerably to about half a part per million. I would say that dehydroabieatic acid is useful just for us on the Pacific coast where we are interested in a lot of pulpmill bioassay work.

Sodium pentachlorophenate by contrast is the more universally available substance. However, I am not a chemist and there may be some disadvantage in using this substance. Alderdice's work, which constituted a PhD thesis, reviewed some forty-three different compounds and he selected sodium pentachlorophenate as being one of the best toxicants to use for reference purposes.

Cecil Inniss

The one problem that I ran into with sodium pentachlorophenate was availability. It seems to be easy to get in commercial forms and it is hard to get in refined form.

John Davis

The compound that we used was an Eastman Kodak product and it might be possible to obtain this fairly universally.

Cecil Inniss

Ed Pessah, what would you use if not DSS?

Ed Pessah

I am toying with the idea of not using anything at all. One of the things that has been occurring to me during this discussion today is the development of a need, for the EPS anyway, to correlate the results from one laboratory to another.

Three years ago when I was in Winnipeg we sent a batch of fish to Halifax. They tested their fish and our fish with sodium chloride. We tested our fish on the same day in Winnipeg. They got 15.5 parts per thousand for a LC-50 while we got 15.2. What suddenly occurred to me was that there are a bunch of fish hatchery people in the business of selling fish whose livelihood depends on selling good quality healthy fish. It occurred to me that if we are using the reference chemical toxicant to just separate the obvious then maybe we really don't need it.

Cecil Inniss

How then would you set a criteria for people? Look at the practical point where a person who has to run a series of bioassays for his industry has purchased his fish with the limited funds allotted to him. If the fish don't seem to be doing well which for rainbow trout is an easy thing to happen, would you say that he discard them?

Ed Pessah

If they were suffering severe mortality he would have to.

Cecil Inniss

If they are obviously not doing too well, but not dying should he discard them?

Ed Pessah

You are asking now for a criteria on when to accept or reject. If we are going to use reference toxicants then they have got to pick up more sublethal responses than the gross ones. If they are not capable of doing that then it is questionable as to why use it at all.

Evan Bertrude

There are a number of conditions that are written into the petroleum refinery regulations that talk about the condition of the fish before they can be used. It is wordy but it mentions that symptoms of disease and abnormalities should not be present during acclimation. There is a statement of the fish having to pass a disease control requirement and other conditions which supposedly have to be met before you can use that fish. Is that not sufficient?

Bridgett de March

I think reference toxicant chemicals have a real place in the search for new bioassay organisms and also in final culture methods. Several people have mentioned that we should be running bioassays on more organisms and we should be able to determine whether strain A is more resistant than strain B, or whether culture methods affect resistance.

Cecil Inniss

I would reinforce that aspect. Pat Hunter found indications using cesium chloride that laboratory reared Gammarus responded differently than field collected Gammarus.

Ray Brouzes

There are two reasons why we need reference toxicants. Firstly, to monitor the quality of suspect water. If I situate an industry on a creek where the water quality is poor and I use that water and discharge into it an iota of a toxicant that is now sufficient to kill the fish and fail the LC-50 test, should I have to clean dirty water before using it? If I had a reference toxicant that could tell me how dirty that water was it would provide a sliding scale to correct for the various water qualities in the industrialized part of Canada. Secondly, with respect to the toxicity emission rate concept, if we are going to play the numbers game and I think we have to, we have to talk about loading rates, BOD etc. So must we move in the field of toxicity and consequently we must have net emission rates. If I have loaded an environment by so many toxic units, I have to know how many units were in the receiving water.

Evan Bertrude

If you want to describe the quality of the incoming water, can you not bioassay that water without the use of a toxicant and compare it with the effluent bioassay?

Ray Brouzes

If I hold a fish stocks using water from pristine stream somewhere in British Columbia and I compare them to those held in streams I have seen in southern Ontario, there will be a difference in health between these two fish populations.

Ed Pessah

Suffice to say that what we want to do is to identify the health and sensitivity of the fish with respect to whatever environment it happens to be in so that you can modify appropriately whatever results you get from some unknown toxicant.

If the dispersant data using DSS illustrated a 20% increase in toxicity and we got an additional 20% increase in toxicity with a commercial oil dispersant it would be important to the company and important to us in Canada as to whether we would allow the use of that commercial product. We need to have some mechanism to determine the relative potency of a new product.

Gary Alexander

I agree with Ed Pessah that we are reaching a point of diminishing returns by trying to prove an obvious difference between two stocks of fish. I think it is premature to begin a cross country testing of one chemical. We have to find out what happens in the laboratory under control conditions with that one chemical and I don't think we have reached that stage.

Peter Hodson

I agree too that before we launch any type of program with reference toxicants it would really pay to quantify firstly the natural variability of the organism, secondly the variability in the method, and thirdly the variability that would be acceptable beyond the realms of the expected.

This was nicely pointed out in your data Jack Klaverkamp that the azide toxicity tests included a range of weights of organisms from about 4.5 to 7.5 grams and the flow rate varied from about two l/gm/day to about one l/gm/day and the toxicity increased by about 50%. Now here you are evaluating a reference toxicant under controlled conditions and yet there is a change in toxicity which could be correlated to the weight change. I think that this type of variability has to be identified before being incorporated in a standard test.

Cecil Inniss

It would be very difficult at this stage to standardize the approach but my thinking was that if we started off with a standardized reference toxicant test, we would have initiated some sort of standardization of approach.

Jack Klaverkamp

So much of the question about the value or use of a standard toxicant revolves around the anticipation or lack of anticipation of a problem. We have one group of people who say "in anticipation of...." for example liver dysfunction, kidney disease or other toxicants in the water, what comes through is "hypothetical". In anticipation of this hypothetical factor that could influence an LC-50, we should do a number of things in the standardization of the test such as controlling the D.O. temperature, flow rates and introduce a reference toxicant.

Or should we? Alternatively in anticipation of disease free, certified fish that are held well, fed well and in anticipation that everything is fine why waste the time on a reference toxicant?

We could spend a lot of time here deciding where we stand in the anticipation game. Gary Alexander has one of the few sets of data that can identify stress induced fish with phenol but not with DSS. This is what is really lacking and I do not see us getting anywhere this afternoon other than seeing who can recall another anticipated event and it is going to stay subjective.

Tom Brydges

Under the I.J.C. there is a committee looking at interlaboratory comparisons in the chemistry area and it was rather shocking to find that in some of the really easy tests there turned up some fairly substantial differences among laboratories. If we just start any sort of nation wide check with a common chemical we may turn up some real horror stories. There is a real advantage to doing something then take a fine tooth comb to what you are doing if you don't get the right answer. This is the purpose of the standard test. We can expect to find some real anomalies and we may be a little shocked. You cannot solve all the problems ahead of time and probably won't.

Ed Pessah

When we have run calibration tests between laboratories we have come remarkably close. We have done some work on a variety of industrial effluents with a variety of laboratories that came up with remarkably similar results. We have also used DSS and sodium chloride so I do not think it will be as bad as you might expect. I think what we have got to decide is what are we going to do if the reference toxicant LC-50 is 20% of a test or series of tests. Before we go on with these tests we have got to know what we are going to do with the results.

Jack Klaverkamp

Let's standardize some things like temperature and dissolved oxygen and collect the data first without deciding what to do with it. Let's define some parameters and do the experiments and then worry about what we are going to do with the data.

Cecil Inniss

The business about getting the same result indicating a good reference toxicant is unacceptable. You should get different results and that will tell you whether it is a good reference toxicant. That is, crude tests do not show great differences. I would like to see a test that shows up a good deal of difference between stocks. When we get these differences we know where to look and why. I am proposing that we assemble a voluntary working group after this session to correspond from their laboratories as to the testing of common reference toxicants. I would invite anyone interested in participating to leave their name and address.

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The 1975 Toxicity Workshop - A Perspective

John C. Davis
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Personally, I was very stimulated by the 1975 Toxicity Workshop as I felt it represented an excellent cross-section of thinking and approaches in use today. I got the impression that considerable progress is being made in researching new techniques and utilizing new test organisms for aquatic toxicology. Indeed, it may be said that much of this work lacks a definitive objective and focus and that various groups and organizations are each pursuing their own goals. I don't think this is necessarily bad as it encourages innovative research. An attempt to impose rigidity on the work by specifying certain test procedures or sole use of 1 or 2 test organisms would do much to destroy the originality of approach that is vital to discovering new procedures. I do not mean to imply that we do not need standardized test procedures and test organisms such as rainbow trout - we do, for both practical and scientific reasons. However, people should be encouraged to work with new procedures and test organisms provided they attempt to relate their results to standard test species. There is no reason why invertebrate bioassays cannot be devised which are fast, economical of test solution, and sensitive as long as the relative sensitivity of the test organism is known in relation to coho salmon or rainbow trout underyearlings.

Reference Toxicants

At the workshop, considerable interest was expressed in the use of reference toxicants. I got the impression that there are a number of ways in which people view reference toxicants as useful. These are:

1) For interlaboratory comparisons on a national scale, where all facilities routinely doing bioassays would test one or more reference toxicants and duly report their results along with the bioassay results.
2) For limited interlaboratory comparisons where several labs using the same or smiliar water supplies may wish to compare results.
3) For regular intralaboratory useage where a worker wants to check the sensitivity of his/her stock from time to time.
4) As a test for sensitivity of test organisms to a specific mode of toxic action - i.e. - what is the sensitivity of the stock to a respiratory or metabolic poison?

In thinking about the above, let us consider each possible use in turn. Item 1 to me seems to be a naive approach owing to the great geographical variance in dilution waters used for bioassays which all differ greatly in hardness, mineral content, dissolved gases, etc. Unless we know precisely how all these variables influence the toxicity of a single reference toxicant we will have a most difficult job trying to compare results across the country. It may be possible, however, to adjust results for comparative purposes by using reference toxicants. Consider the following hypothetical situation using two reference toxicants that have a similar mode of toxic action but different LC50's.

Laboratory	mg/l LC50-Toxicant #1	mg/l LC50-Toxicant #2	Ratio 2/1
Vancouver	10	100	10
Winnipeg	17	170	10
Toronto	19.5	195	10
Halifax	6	60	10

Obviously, the above are highly idealized data, but we might be able to achieve standardization by considering toxicity ratios of a reference substance with an unknown. What we need is a few volunteers across Canada to test this hypothesis.

Items 2 and 3 to me, seem to be practical uses for reference toxicants. In the Pacific region we have a number of labs, both government and private, doing bioassays routinely to test for compliance with Provincial and Federal regulations. Obviously, one wishes to know if results from these labs are comparable, within reasonable limits, so that there is no inequity in the system. We concluded in our published work, that the procedure was useful when all the labs followed detailed bioassay guidelines and used identical or similar water sources. Similarly, reference toxicants were thought useful for detecting large variations in sensitivity of one's test stock over a period of time. No one has really tested this assumption however, and it can be reasonably argued that a good diagnostic routine health check might turn up incidences of weakened fish or low-level disease. One problem here is that there are relatively few facilities available to provide such diagnostic checks on a routine basis, and I suspect that their procedures would require standardization. Obviously, we need some experimental studies which test the ability of reference toxicants to detect low level disease or presence of other stresses.

Item 4 is based on the assumption that a reference toxicant is available whose mode of toxic action is known. Ideally, it would be desirable to utilize a reference toxicant whose mode of action resembled that of the test substance being bioassayed. The problem here, of course, is that we usually don't know the mode of toxic action of the test substance, or in some instances, the reference toxicant. It is likely that progress in this area will be very slow. Certainly, with the vast array of potentially dangerous substances entering the aquatic environment today, there is precious little time to screen all these substances, let alone determine the mode of toxic action for each one of them.

In summary,there would appear to be several potential uses for reference toxicants,and our objectives in using them must be carefully thought out without dogmatically adopting the procedure at this time. More work is required and co-operative research among laboratories is needed. Appendix I lists possible reference toxicants and the desirable properties which require evaluation.

Sublethal Effect Studies

I think all of us recognize the short-comings of acute toxicity tests and look to sublethal test procedures as a means of deriving "safe" tolerance limits for pollutants. From the proceedings of this workshop, as well as the expanding scientific literature, it is evident that considerable research emphasis is being placed on sublethal studies. Again, as in most new work, people seem to lack a unified focus or objective in their work. The business of acute toxicity tests is to provide numbers (concentrations) which define threshold levels. I think sublethal tests should do the same thing, with the advantage being that they are more sensitive, are more representative of long-term hazards and hopefully, closer to the "real world" in experimental design. Obviously, we have a long way to go with our procedures to make the tests truly representative of the dynamic conditions in natural waters.

In my written paper (enclosed) I have illustrated one simple-minded way in which sublethal studies can be summarized to produce receiving water criteria for kraft pulp mill waste. Clearly, when enough different studies have been done, with a variety of conditions and various life stages, such an approach may be useful. One advantage of this approach is that if incipient sublethal threshold concentrations are expressed as fractions of the acute LC50 value, then the acute toxicity test becomes useful for predicting sublethal effect levels. The problem inherent in this approach is one of time and manpower - can we possibly achieve this goal for every major pollutant? I suspect not! What we really need is a simple sensitive test which defines the incipient sublethal threshold concentration for the toxicant in question. Even better, might be a quick, predictive sublethal test such as the enzyme activity bioassay Dr. Peter Hodson described. Current research should be aimed at describing sensitive sublethal tests, which are both rapid and practical so that a range of research tools is available for use as the special characteristics and species composition of an area dictate. Receiving water criteria could be based on these incipient threshold concentrations with a "safety factor" for additional protection.

People ask if there is one or several sublethal test procedures that look especially useful. I tend to favour the simple tests on the basis that procedures involving complex equipment and long exposure times may be impractical for generalized use and prone to many sources of variation. Dr. John Sprague (1975 - paper given at Pacific Science Congress, Vancouver, B.C.) did an analysis of sublethal methodology summarized in the 1972 U.S. E.P.A. "Blue Book", Water Quality Criteria. He concluded that we are still a long way from having a scientific basis for good water quality criteria and that the most promising procedures appeared to be sublethal tests involving fish reproduction, fish egg development and studies with invertebrates (Daphnia) and possibly algae. Dr. Sprague is a strong advocate of adopting test organisms with a rapid generation time to facilitate reproductive and developmental studies. He was critical of much laboratory physiology as "yielding few hard numbers" and pointed out that results of some approaches (e.g. growth studies) were disappointing. John felt that a range of experiments at the tissue level (blood studies, histology, enzyme assays) remained largely unexplored and that sensitive behavioral tests such as avoidance studies were underrated. In general, the area of sublethal reproduction and development studies seems the most promising. I see no objection to working with a variety of fish and invertebrate species, provided results are related to standard test organisms or threatened populations in specific receiving waters. Fish and invertebrate species being studied by people attending the workshop are summarized in Appendix I.

Conclusions

In retrospect I felt the Toxicity Workshop provided a very good cross-section of technology, approaches and concerns of toxicologists working in the field today. Obviously, there is a great deal being done and progress is being made. We are reaching the point where the objectives of our work and our goals need definition. There is a need to continue discussions of this type in future and to devote some of our effort to planning and objective definition as a guide to future work.

Please feel free to comment or disagree with the above thoughts - they just reflect my point of view and I may well have missed a key point or misinterpreted someone's paper.

John Davis
November 20, 1975

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Appendix I

Possible Reference Toxicants

D.S.S. p,p' DDT
Phenol
Sodium azide
Copper sulfate
Dehydroabietic acid
Sodium pentachlorophenate
Lethal temperature
NaCl

Possible Desirable Properties of Reference Toxicants

1) Alderdice (1963)
    - high solubility in H_2O
    - virtually complete ionization in solution at pH 7.0 or higher (excepting organics)
    - stability in solution at pH 7.0 - 8.5
    - availability as a dry solid of high purity
    - facility for quantitative determination
    - high toxicity to fish (in low concentrations)

2) Klaverkamp (1975)
    (see Dr. Klaverkamp's paper enclosed)

Fish & Invertebrate Species Studied by Workshop Participants

Fish

- rainbow trout
- Atlantic salmon
- sockeye salmon
- coho salmon
- pink salmon
- chum salmon
- flagfish
- fathead minnows
-

Invertebrates

- *Daphnia*
- atlantic lobster larvae
- mosquito larvae
- *Hyallela*
- fresh water snails
- mayfly larvae
- damselfly larvae
- chironomid larvae

- yellowtail rockfish (Pacific-marine)
- goldfish

- *Gammarus*

Memo to: Dr. John C. Davis 26 November, 1975
From: M. Waldichuk

Some points that come to mind with respect to aquatic toxicity and bioassays:

1. With all their short-comings, acute bioassays over 96 hour exposures will continue to be used for some time as management tools. A 96 hr LC50 is still a pretty important number, even if it is often used with some sort of arbitrary application factor.
2. We need more long-term studies that will relate the incipient lethal threshold to the LC50 In this respect, some studies have been carried out on incipient lethal levels of DDT and PCB's at the Gulf Breeze Laboratory of EPA, and they appear to be quite close to the application factor (0.01) generally used for these organochlorines.
3. Your workshop was probably very much freshwater-oriented. As I've said in the past, we need more bioassay data obtained in parallel experiments with freshwater and seawater systems, preferably using the same species (e.g. coho) acclimated to the particular water. As we have found with boron and some of the"neutrals" fractions from KME, we may have higher toxicity for some substances in sea water than in fresh water because of some peculiar mode of toxic action. We need to know more about the mechanism of this "increased seawater toxicity". Clearly, we should not be using freshwater toxicity data to develop criteria for sea water.

4. While it may be important to refer tests with such organisms as invertebrates to standard test species, e.g. coho or rainbow trout, I think it is essential to run tests on series of metals, for example, with the same sensitive species, whether it is brown shrimp or oyster larvae. Only in this way can one obtain relative toxicities of a series of similar substances. I found particular difficulty in obtaining consistent information on metal toxicities in sea water. I finally used a set of data on the effects of some of the metals on the larvae of the eastern American oyster, *Crassostrea virginica*, done at the EPA laboratory in Narragansett.

5. It is obvious that we have to move more and more into sublethal bioassays, if we are going to provide information that's meaningful and applicable to the "real world". However, scientists and laboratories tend to steer away from such studies because of the high degree of sophistication often involved with advanced physiological-type tests. We need more of the simple sublethal tests which any laboratory with a few water tanks and microscopes can apply. This is particularly important for laboratories in developing countries. A few suggested "simple" sublethal tests:

   (a) Regeneration rate of byssal threads on mussels under different concentrations of pollutant.
   (b) Growth rate of hydroids. Some of this type of work was initiated with respect to metals effects by Karbe in Hamburg. It is also being pursued by the Natural Environmental Research Council Laboratory in Plymouth, U.K.
   (c) Avoidance reactions in behaviour studies.
   (d) Reproduction rate in short life-span organisms.

(e) Effects on eggs and larvae on those organisms that can be made to spawn the year around.

(f) We need to know more about toxicities of mixtures of substances.

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Guest Speakers

- Dr. J.B. Sprague
- Dr. J.C. Davis
- Dr. P. Doudoroff

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